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MECHANISMS AND REGULATION OF THE POLYPHOSPHATE/FACTOR XII-DRIVEN CONTACT SYSTEM IN THROMBOSIS AND HEMOSTASIS

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MECHANISMS AND REGULATION OF THE POLYPHOSPHATE/FACTOR XII-DRIVEN CONTACT SYSTEM IN THROMBOSIS AND HEMOSTASIS

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ABSTRACT

Blood coagulation leading to fibrin formation is essential to prevent loss of blood (hemostasis), but can also contribute to occlusion of vessels (thrombosis). Thrombosis causes pulmonary embolism, myocardial infarction and stroke, which are together the most common cause of death in the developed world. Current anticoagulation therapy for prevention or treatment of thromboembolic events is sufficient, however results in an increase in potentially life threatening bleedings.

Two distinct pathways initiate fibrin formation: one pathway is triggered by tissue factor exposed on damaged vessel walls (extrinsic pathway) and the other by blood-borne factors (intrinsic pathway). The intrinsic pathway starts by activation of blood coagulation factor XII. The findings that factor XII appears to be involved in pathologic thrombus formation, and that factor XII deficiency is not associated with abnormal bleeding has led to a significant interest for factor XII in the scientific community. Inhibition of factor XII seemed to offer a selective and safe strategy for preventing thrombotic diseases.

Coagulation factor XII becomes activated by contact with negatively charged surfaces. In previous studies it is shown that platelets initiated factor XII-driven coagulation *in vivo* and this activation is driven by polyphosphate. Polyphosphate is an inorganic polymer that has been identified as a potent procoagulant and proinflammatory mediator *in vitro* and *in vivo*. Polyphosphate initiated fibrin formation by the factor XII-driven intrinsic pathway of coagulation. An infusion of polyphosphate induced lethal pulmonary emboli in mice, while a deficiency in factor XII or pharmacological inhibition of factor XII activity protected animals from this pathological clot formation. These data showed that polyphosphate driven factor XII activation is a necessary mechanism in platelet-driven thrombosis *in vivo*.

In this thesis we localize polyphosphate on activated platelets and establish and characterize recombinant tools to neutralize procoagulant polyphosphate activities based on a bacterial exopolyphosphatase. Exopolyphosphatase is an enzyme, which specifically breaks down polyphosphate. Using mutagenesis we identify a specific polyphosphate-binding mutant based on the binding domain of the exopolyphosphatase, resulting in a dual strategy of binding and degrading polyphosphate to interfere with procoagulant polyphosphate activities. Both strategies block the ability of polyphosphate to activate factor XII in different *in vitro* assays. Furthermore pretreatment of mice with exopolyphosphatase or the specific polyphosphate-binding mutant interferes with arterial thrombosis and protects from lethal pulmonary thromboembolism induced by platelet activity. Moreover the bleeding time of these mice are completely normal, showing that targeting of polyphosphate does not interfere with hemostasis. Finally, we use the specific polyphosphate-binding mutant as a probe to analyze polyphosphate on cells and in human samples. We present the first assay to analyze polyphosphate in human platelet-rich plasma offering the opportunity of analyzing a possible thrombotic biomarker in future clinical trials.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers:

I. **Polyphosphate nanoparticles on the platelet surface trigger contact system activation**

Johan J.F. Verhoef[*], Arjan D. Barendrecht[*], Katrin F. Nickel[*], Kim Dijkxhoorn, Steven de Maat, Ellinor Kenne, **Linda Labberton**, Owen McCarty, Raymond Schiffelers, Antoni P. Hendrickx, Huub Schellekens, Marcel H. Fens, Thomas Renné, Coen Maas

Submitted manuscript

[*] = equal contribution

II. **Neutralizing blood-borne polyphosphate *in vivo* provides safe thromboprotection**

Linda Labberton, Ellinor Kenne, Andy T. Long, Katrin F. Nickel, Antonio Di Gennaro, Rachel A. Rigg, James S. Hernandez, Lynn M. Butler, Coen Maas, Evi X. Stavrou, Thomas Renné

Nature Communications 2016;7:12616 doi: 10.1038/ncomms12616

III. **A flow cytometry-based assay for procoagulant platelet polyphosphate**

Linda Labberton, Andy T. Long, Sandra J. Gendler, Christine Snozek, Evi X. Stavrou, Katrin F. Nickel, Coen Maas, Stefan Blankenberg, James S. Hernandez, Thomas Renné

Submitted manuscript

IV. **The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis**

Katrin F. Nickel, Göran Ronquist, Florian Langer, **Linda Labberton**, Tobias A. Fuchs, Carsten Bokemeyer, Guido Sauter, Markus Graefen, Nigel Mackman, Evi X. Stavrou, Gunnar Ronquist, Thomas Renné

Blood 2015;126(11):1379-1389

Additional papers by the author that are not included in this thesis:

Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions

Anna Sala-Cunill, Jenny Björkqvist, Riccardo Senter, Mar Guilarte, Victoria Cardona, Moises Labrador, Katrin F. Nickel, Lynn M. Butler, Olga Luengo, Parvin Kumar, **Linda Labberton**, Andy T. Long, Antonio Di Gennaro, Ellinor Kenne, Anne Jämsä, Thorsten Krieger, Hartmut Schlüter, Tobias A. Fuchs, Stefanie Flohr, Ulrich Hassiepen, Frederic Cumin, Keith McCrae, Coen Maas, Evi X. Stavrou, Thomas Renné

Journal of Allergy and Clinical Immunology 2015;135(4):1031-43

New agents for thromboprotection. A role for factor XII and XIIa inhibition

Linda Labberton, Ellinor Kenne, Thomas Renné

Hamostseologie 2015;35(4):338-50

The polyphosphate/factor XII pathway in cancer-associated thrombosis: novel perspectives for safe anticoagulation in patients with malignancies

Katrin F. Nickel, **Linda Labberton**, Andy T. Long, Florian Langer, Tobias A. Fuchs, Evi X. Stavrou, Lynn M. Butler, Thomas Renné

Thrombosis Research 2016;141(suppl 2):S4-7

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	One-way analysis of variance
APC	Activated protein C
aPTT	Activated partial thromboplastin time
ASO	Antisense oligonucleotides
ATP	Adenosine triphosphate
BK	Bradykinin
bw	Bodyweight
B_2-receptor	B2 bradykinin receptor
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CD42b	Platelet glycoprotein Ib alpha chain
CD62P	P-selectin
CS	Chondroitin sulfate
CTI	Corn trypsin inhibitor
C1INH	C1 esterase inhibitor
DAPI	4',6 diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DS	Dermatan sulfate
DXS	Dextran sulfate
EDAC	1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide
EDTA	Ethylediaminetetraacetic acid
ETP	Endogenous thrombin potential
Fe	Iron
$FeCl_3$	Ferric chloride
FV	Plasma coagulation factor V
FVa	Activated plasma coagulation factor V
FVII	Plasma coagulation factor VII
FVIIa	Activated plasma coagulation factor VII
FVIII	Plasma coagulation factor VIII
FVIIIa	Activated plasma coagulation factor VIII
FIX	Plasma coagulation factor IX
FIXa	Activated plasma coagulation factor IX
FX	Plasma coagulation factor X
FXa	Activated plasma coagulation factor X
FXI	Plasma coagulation factor XI
FXIa	Activated plasma coagulation factor XI
FXII	Plasma coagulation factor XII
FXIIa	Activated plasma coagulation factor XII
FXIIa	Activated plasma coagulation factor XII light chain fragment
GDP	Guanosine diphosphate
GPPA	Guanosine pentaphosphate phosphohydrolase
GTP	Guanosine triphosphate

HK	High molecular weight kininogen
HS	Heparan sulfate
Ion exc	Anion exchange
IP6K1	Inositol hexakisphosphate kinase 1
LC	Long-chain
MFI	Mean intensity of fluorescence
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
Mn^{2+}	Manganese
mTOR	Mechanistic target of rapamycin
NETs	Neutrophil extracellular traps
Ni^{2+}	Nickel
OSCS	Over sulfated chondroitin sulfate
PAMAM	Polyamidoamine dendrimers
PC	Prostate cancer
PC3	Human prostate cancer cell line
Phen/Chl	Phenol-chloroform
PK	Plasma prekallikrein
PL	Phospholipids
polyP	Polyphosphate
ppGpp	Guanosine tetraphosphate
PPK1	Polyphosphate kinase 1
PPK2	Polyphosphate kinase 2
PPP	Platelet-poor plasma
pppGpp	Guanosine pentaphosphate
PPX	Exopolyphosphatase of *E.coli*
PPX_Δ12	Exopolyphosphatase of *E.coli* lacking domain 1 and 2
PPX_Δ12-Alexa488	PPX_Δ12 covalently coupled to amine-reactive Alexa488
PRP	Platelet-rich plasma
PSP	Alkaline phosphatase
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SC	Short-chain
TAFI	Thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
trap6	Thrombin receptor activator peptide 6
UHRAs	Universal heparin reversal agents
VTC	Vacuolar transporter chaperone
vWF	Von Willebrand factor
w/o	Without
WT	Wild type
Zn^{2+}	Zinc

INTRODUCTION

Blood is a liquid that circulates in our vascular system to provide the transport of oxygen, the distribution of nutrients and to remove carbon dioxide and other waste products. Furthermore, is the circulation involved in the defense against foreign organisms. To sustain these functions, blood has to be kept circulating in an intact and functional vascular system. However, when injury happens the blood has to clot to prevent extensive blood loss. The process that regulates the blood flow in case of vessel injury is called hemostasis (from the ancient Greek where *hema* means blood and *stasis* halt). Hemostasis is dependent on a fine balance between bleeding and clotting and in a delicate balance such as this, mistakes may occur, leading to events such as thrombosis or hemorrhage. By learning more about the different processes involved in hemostasis, many of these events may be prevented in the future. My contribution to the science of hemostasis, especially to the prevention of thrombosis will be discussed in this thesis.

1. HEMOSTASIS

Hemostasis is the process by which the blood changes from a liquid form to a solid state. If bleeding occurs three main mechanisms lead to a series of events. These mechanisms are vasoconstriction, platelet activation and blood coagulation and they are divided in two phases, primary and secondary hemostasis (**Figure 1**). Primary hemostasis is the formation of a platelet plug, however this patch is temporary and will be replaced by a clot during the stage of secondary hemostasis.

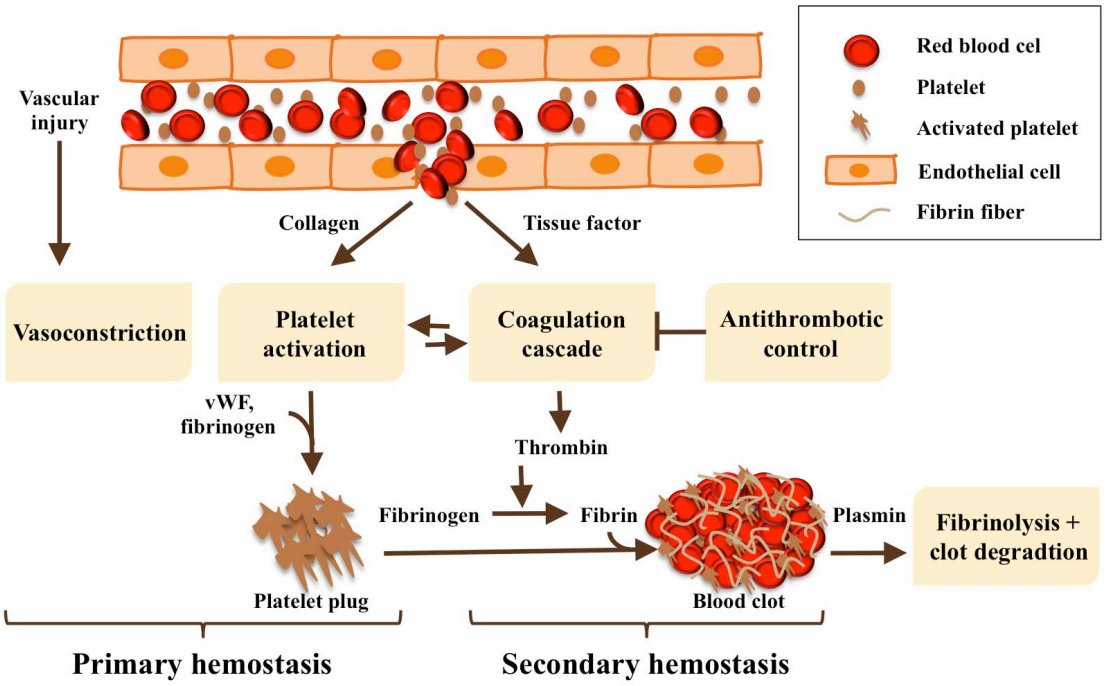

FIGURE 1: Components of hemostasis.
Schematic representation of events happening in primary and secondary hemostasis. Following the initial vasoconstriction, platelet plug formation limits the blood loss and the activation of the coagulation cascade results in fibrin production that strengthens the blood clot. Finally, activation of the antithrombotic control and the fibrinolytic pathway leads to prevention of uncontrolled clot formation.

1.1 Primary hemostasis

Primary hemostasis involves formation of the primary platelet plug and starts with vasoconstriction to keep blood loss limited. Immediately after vascular injury vasoconstriction occurs initiated by the pain receptors and the effect will continue by release of chemical substances from the endothelial cells and platelets. The degree of vasoconstriction is dependent on the amount of tissue damage.

Upon injury the subendothelial matrix will get exposed and circulating platelets will start to adhere directly or indirectly via von Willebrand Factor (vWF). When platelets adhere they will become activated by collagen and start to recruit and activate additional platelets. Key receptors in these events are the platelet receptors that bind to collagen (integrin α2β1 and glycoprotein VI) and those that bind to vWF (glycoprotein Ib). Activated platelets undergo a variety of changes where shape change, degranulation and phosphatidylserine exposure are some examples. Furthermore the fibrinogen receptor (integrin αIIbβ3) changes conformation, which allows fibrinogen to forms bridges between activated platelets. The platelet plug is enough to stop bleeding from minor trauma, however with more severe damage, the primary platelet plug must be stabilized. This is accomplished through secondary hemostasis (Clemetson, 2012).

1.2 Secondary hemostasis – coagulation

The main product of the coagulation system is thrombin, which transforms fibrinogen into fibrin strands that stabilize the platelet plug. In the classical coagulation cascade, coagulation is divided into the intrinsic, extrinsic and common pathway (**Figure 2**). Triggers of the intrinsic pathway are found in blood, whereas the extrinsic pathway needs a trigger present in extravascular tissue (Geddings & Mackman, 2014).

1.2.1 Extrinsic pathway

The extrinsic pathway leading to the formation of thrombin is initiated when tissue factor (TF) appears at vascular injury sites. On its own, TF has no enzymatic function but serves as a cofactor and activates blood-clotting factor VII (FVII) to factor VIIa (FVIIa). The complex of TF and FVIIa generates, in the presence of phospholipids and calcium minor amounts of activated factor X (FXa). Activated factor X forms a complex with activated factor V (FVa) that cleaves prothrombin leading to thrombin. The low concentration of formed thrombin is not enough for fibrin formation, though it can activate platelets and convert factor V and factor VIII to their active forms (Mackman, 2009).

1.2.2 Intrinsic pathway

The intrinsic pathway of coagulation is controlled by the following three plasma proteins; factor XII (FXII), plasma prekallikrein (PK) and high molecular weight kininogen (HK). Exposure of blood to a negatively charged surface initiates the pathway and activates FXII. Activated factor XII (FXIIa) forms active FXI (FXIa) by enzymatic cleavage of factor XI (FXI) that further promotes coagulation via calcium dependent activation of factor IX (FIX).

Activated FIX (FIXa) together with activated factor VIII (FVIIIa), phospholipids and calcium generates FXa. FXa complexes with the cofactor FVa and this complex converts prothrombin into thrombin (Gailani & Renne, 2007). Activated FXII can also convert PK to kallikrein. Kallikrein can then activate new FXII zymogens and this feedback loop amplifies FXII activation (Bjorkqvist et al., 2013a).

1.2.3 Common pathway

Formed FXa and its cofactor FVa start the common pathway of coagulation leading to the conversion of fibrinogen to fibrin by thrombin. Thrombin will additionally activate factor XIII that results in a stable covalently cross-linked fibrin network in which platelets and blood cells are captured and a blood clot is formed (Dorgalaleh & Rashidpanah, 2016). Furthermore, thrombin converts the cofactors factor V and factor VIII into active cofactors, which facilitates the formation of more FXa/FVa and FIXa/FVIIIa complexes on a phospholipid surface. As the amount of complexes increases, big amounts of thrombin are generated, yielding a polymerized fibrin clot (Versteeg et al., 2013). Thrombin can also amplify its own generation by activating FXI, showing that the formation of FXa can be accelerated, independently of FXII (Gailani & Broze, 1991).

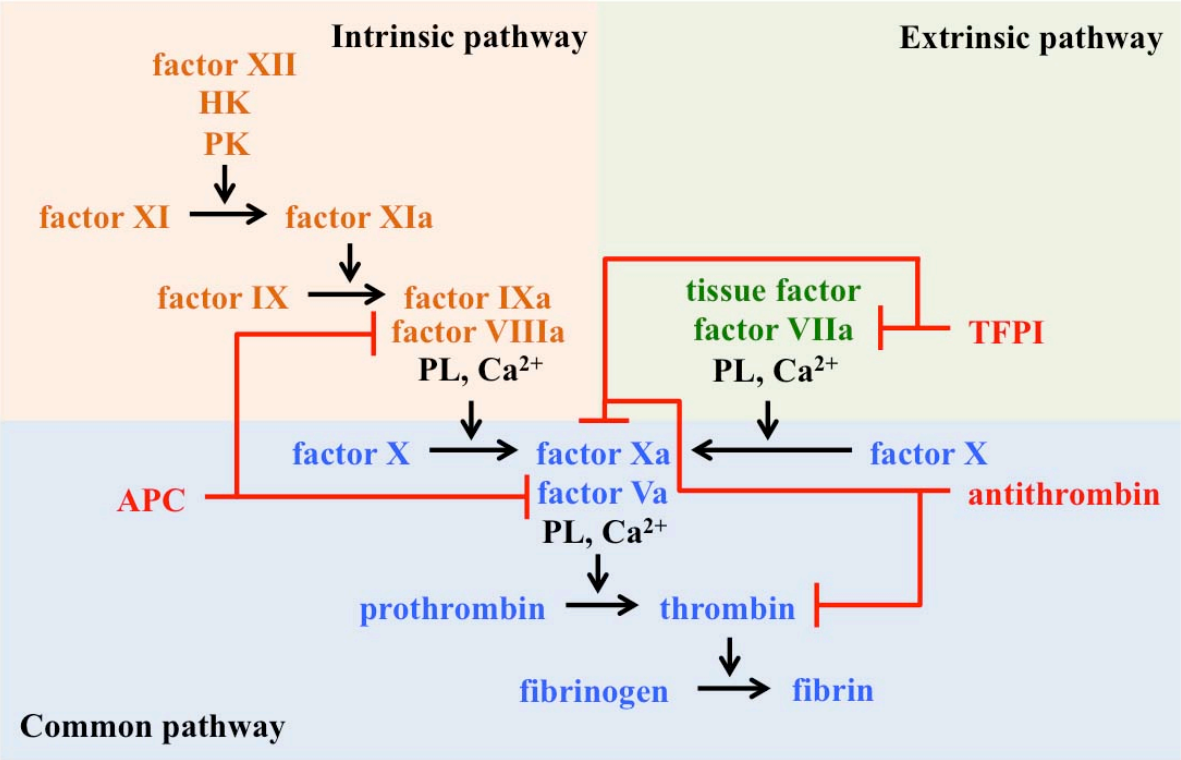

FIGURE 2: The cascade model of coagulation.

Orange, green and blue coagulation factors belong to the intrinsic, extrinsic, and common blood coagulation pathways, respectively, and red proteins are part of the antithrombotic control. Black horizontal arrow-headed lines indicate the catalytic conversion from zymogens to their active forms, and red bar-headed lines indicate the inhibition of the active proteases. APC= activated protein C, TFPI= tissue factor pathway inhibitor, Ca^{2+}= calcium and PL= phospholipids.

1.3 Natural inhibitors of coagulation

The coagulation cascade works fast and amplifies itself to prevent blood loss from the site of injury, however this explosive system needs dampening to prevent widespread and uncontrolled clot formation away from the site of injury. Thrombin plays an important roll also here. Formed thrombin will bind to thrombomodulin, where it will be presented to protein C, after which protein C will become activated (APC). APC and its cofactor protein S can then inactivate FVa and FVIIIa. Additionally, the circulating protease inhibitor tissue factor pathway inhibitor (TFPI) can reversibly inhibit FXa and the TF-FVIIa complex to inhibit coagulation. Another circulating plasma protein, antithrombin, controls coagulation by inhibiting functions of thrombin, FIXa and FXa (Versteeg et al., 2013).

1.4 Fibrinolysis

Fibrin is one of the main players in hemostasis. It is the final product of the coagulation cascade and the substrate for fibrinolysis. Fibrinolysis is the process where a formed clot is broken down to maintain hemostatic harmony. Like the coagulation cascade, fibrinolysis is controlled by a series of cofactors, inhibitors, and receptors. Plasmin is the main protease that acts to dissolve fibrin clots into fibrin degradation products. Plasminogen is converted into plasmin by one of two circulating proteases; tissue-plasminogen activator or urokinase-plasminogen activator. The main inhibitors of fibrinolysis are plasminogen activator inhibitor-1 and α2-antiplasmin, which bind to the plasminogen activators or plasmin, respectively.

Thrombin-activatable fibrinolysis inhibitor (TAFI) reduces fibrinolysis when it becomes activated by thrombomodulin-associated thrombin. This inhibitory effect is based on the fact that TAFI removes C-terminal residues on fibrin. These residues are important for the activation and binding of plasminogen to fibrin, therefore TAFI strongly reduces the activation of plasminogen on the fibrin surface, slows plasmin generation and stabilizes the fibrin clot (Chapin & Hajjar, 2015).

1.5 Thrombosis

Coagulation is needed for hemostasis, however excessive coagulation leads to thrombosis. Thrombosis is a formed blood clot in a vessel, which obstructs the blood flow resulting in a major medical disease. Thrombosis occurs in the arteries or veins and is a leading cause of death in the developed world. Understanding the processes behind thrombosis is crucial for developing more effective and saver antithrombotic drugs (Mackman, 2008). Virchow's triad describes the three main features to explain the pathophysiological mechanisms leading to thrombosis. These three features are thought to be abnormalities in blood composition (function of plasma proteins and circulating blood cells), irregularities in vessel wall components (endothelial injury or dysfunction) and changes in blood flow (stasis or turbulence, Wolberg et al., 2012). Thrombosis is commonly divided into arterial and venous thrombosis and they differ in the way of treatment. However, the primary pathophysiology between the two seems to be linked. The risk factors for the two diseases are the same such as

age, surgery, obesity, cancer, diabetes mellitus, hypertension, hypertriglyceridemia, and metabolic syndrome (Prandoni, 2009).

In arterial thrombosis an artery is occluded by a blood clot. The rupture of an atherosclerotic plaque is the primary trigger for arterial thrombosis. When a plaque ruptures platelets are recruited, activated and platelet-rich thrombi will be formed at the rupture site resulting in rapid growth of thrombi. An alternative cause of arterial thrombosis is atrial fibrillation, which causes stasis of blood within the atria of the heart. Arterial thrombosis occurs mainly in coronary and carotid arteries where it will result in a myocardial infarction or stroke, respectively (Berger et al., 2006).

Deep vein thrombosis mostly occurs in the large veins of the leg. Pulmonary embolism is the result of embolization of a part of a thrombus in the leg to the lungs. Collectively, these diseases are termed venous thrombosis. Venous clots are formed under low shear stress, they are rich in red blood cells and fibrin and are called “red clots” (in contrast to the “white” platelet-rich thrombi in arteries, Mackman, 2012).

1.6 Bleeding disorders

When hemostasis does not function properly bleeding disorders may present in a variety of ways. Bleeding can be caused by malfunctions of primary hemostasis (platelet function disorders, von Willebrand disease), secondary hemostasis (hemophilia A and B), fibrinolysis or disorders in vascular formation and connective tissue. It can be inherited or acquired and the clinical presentation varies from slight bruising to life-threatening bleedings. Treatment consists of vitamin K, plasma or platelets transfusion and blood derivatives (including single and multiple factor concentrates, DeLoughery, 2015).

2. CONTACT SYSTEM

The contact system is an enzymatic cascade that is prothrombotic by activating the intrinsic pathway of coagulation and proinflammatory by initiating the formation of the inflammatory mediator bradykinin (BK, **Figure 3**). Contact system proteins circulate in the blood stream or assemble on various cardiovascular cells such as platelets, leukocytes and endothelial cells (Renne et al., 2000). The name contact system is because they require contact to negatively charged surfaces for zymogen activation.

2.1 Proteins of the contact system

The contact system consists of the non-enzymatic cofactor HK and the proteases PK, FXI, FXII and the major inhibitor of FXIIa and kallikrein, C1 esterase inhibitor (C1INH, Colman & Schmaier, 1997).

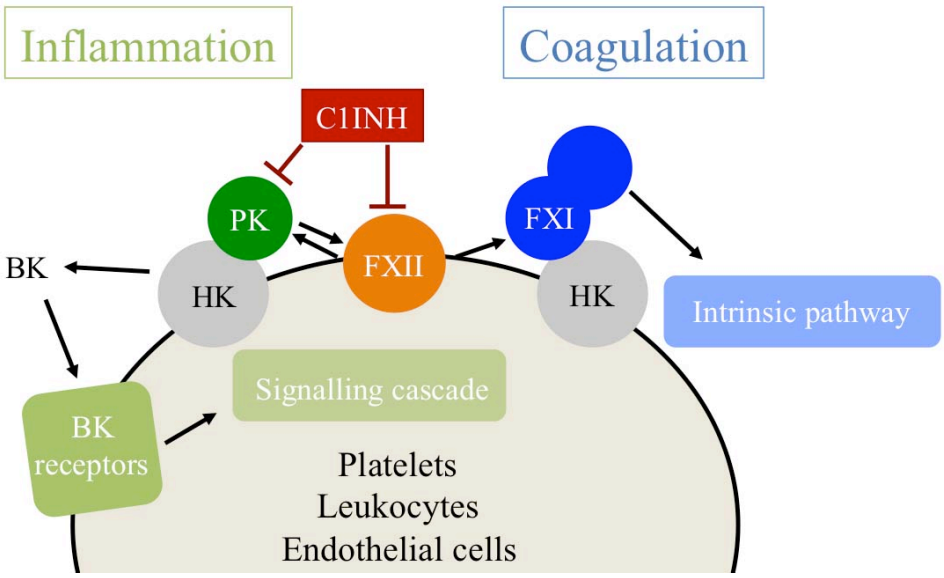

FIGURE 3: The FXII-driven contact system.
Contact with negatively charged surfaces activates the zymogen FXII on the surface of various cardiovascular cells. The FXII-driven contact system is at one side a proinflammatory plasma protease cascade by activating the kallikrein-kinin system leading to generation of the vasoactive peptide bradykinin (BK) by kallikrein mediated cleavage of high molecular weight kininogen (HK). On the other side, the FXII-driven contact system activation generates fibrin formation through the intrinsic pathway of coagulation. C1 esterase inhibitor (C1INH) is the central inhibitor of the contact system. Modified with permission (Renne et al., 2012).

2.1.1 High molecular weight kininogen (HK)

HK, also called Fitzgerald factor, is critical for the assembly of the contact system. HK is not enzymatically active but it is necessary as cofactor for the binding of PK and FXI to cell surfaces. HK is also the precursor of BK. Mice lacking plasma HK display a significantly prolonged time to artery occlusion, suggesting that plasma HK contributes to arterial thrombosis in mice (Merkulov et al., 2008). Furthermore suffer HK deficient mice from less edema formation and have reduced local inflammatory responses without an increase in bleeding. Thus HK appears to be important in thrombus formation and inflammation but dispensable for hemostasis (Langhauser et al., 2012).

2.1.2 Plasma prekallikrein (PK)

PK, Fletcher factor, is a glycoprotein that is secreted as a single chain zymogen and circulates mostly as a complex with HK (Colman & Schmaier, 1997). PK shares high homology with FXI however FXI is a dimer whereas PK is a monomer. The major physiological activator of PK is FXIIa, which cleaves a single peptide bond to generate kallikrein (Bjorkqvist et al., 2013a). PK deficiency in humans or mice does not reveal a prolonged bleeding time. Though PK-deficient mice are protected from occlusion in arterial thrombosis models and selective knockdown of PK inhibits venous thrombus formation, demonstrating that PK deletion can inhibit thrombus formation without affecting hemostasis (Bird et al., 2012, Revenko et al., 2011).

2.1.3 Factor XI (FXI)

Factor XI, plasma thromboplastin antecedent, is a dimeric glycoprotein consisting of two identical polypeptides and circulates as a non-covalent complex with HK. Factor XIIa triggers fibrin formation via activation of FXI in the intrinsic pathway of coagulation. Increased FXI levels have been associated with higher risk for venous thrombosis while deficiency of FXI results in a mild to moderate bleeding disorder (Renne et al., 2002). The finding that deficiency in FXI results in bleeding and that deficiency of HK, PK and FXII does not, suggests that an alternative mechanism for the activation of FXI exists. And indeed, FXI is not only required for the initiation of coagulation, but additionally functions to support hemostasis by feedback activation of FXI by thrombin. This revised model of coagulation reveals a role for FXI in the propagation of clot growth after TF-dependent initiation (Gailani & Broze, 1991).

2.1.4 Factor XII (FXII)

FXII, Hageman factor, is produced by the liver as a single chain zymogen of 80 kDa. Activated FXII leads to fibrin production by the intrinsic pathway of coagulation via activation of FXI, and to formation of BK by the proinflammatory kallikrein-kinin system. Binding to negatively charged surfaces by its heavy chain activates FXII. Not only surface binding activates FXII also kallikrein can activate the FXII zymogen, hereby amplifying FXIIa formation (Maas & Renne, 2012). Reduction of the disulphide bridge from FXIIa releases the protease domain from the surface binding heavy chain, resulting in β-FXIIa, a 28 kDa product (**Figure 4**). β-FXIIa retains its proteolytic activity towards PK but has not the capacity to activate FXI, in contrast to FXIIa, thereby playing no role in the intrinsic pathway of coagulation (Stavrou & Schmaier, 2010). Activated FXII is also able to bind fibrin and can strengthen the clot structure. FXIIa-initiated fibrin formation gives stabilization to the thrombus formed away from the vascular TF, to prevent embolization (Kuijpers et al., 2014a). However, similarities are seen in proteins of the contact- and the fibrinolytic-system and FXIIa can also convert plasminogen to plasmin, promoting fibrinolysis (Konings et al., 2015).

2.1.5 C1 esterase inhibitor (C1INH)

The main function of the serine protease inhibitor C1INH is the inhibition of the complement system to prevent spontaneous activation. Additionally, C1INH is the most important endogenous inhibitor of FXIIa and kallikrein in the contact system. FXIIa and kallikrein cleave the reactive center of C1INH, which induces a rearrangement of C1INH and formation of a covalent binding between C1INH and the protease. The newly formed complex results in an inactive protease (Davis et al., 2010).

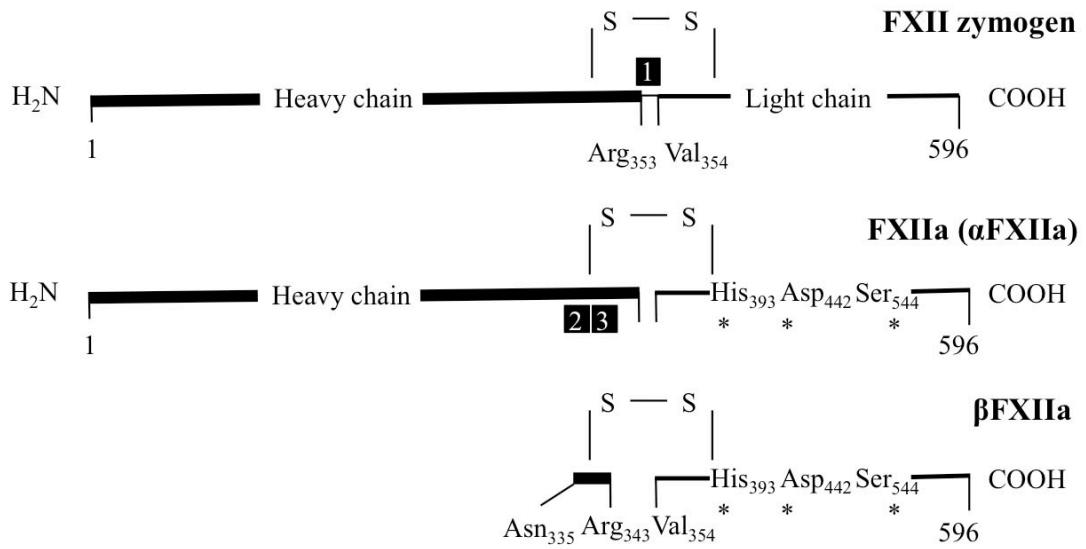

FIGURE 4: The structure of FXII.
The heavy chain of zymogen FXII contains the surface binding region of FXII and the light chain harbors the enzymatic site. When FXII gets activated the peptide bond between Arg_{353} and Val_{354} 1 is cleaved and FXII zymogen converts to the active enzyme, FXIIa. The heavy and light chain are still connected via a disulfide bridge, asterisks indicate the FXIIa catalytic triad. Further proteolysis of FXIIa by kallikrein at peptide bonds Arg_{334}-Asn_{335} 2 and Arg_{343}-Leu_{344} 3 releases the light chain fragment (βFXIIa). β-FXIIa retains its proteolytic ability to activate PK but is not longer able to bind to activate FXI. Modified with permission (Labberton et al., 2015).

2.2 Effects of contact activation

In addition to coagulation and inflammation the contact system has a role in fibrinolysis and angiogenesis. Activation leads to FXII-driven plasmin formation, which contributes to fibrinolysis, tissue remodeling, and wound healing. Moreover contact activation can start the classical pathway of the complement system (Maas et al., 2011). Relevance of contact activation for maintaining hemostatic capacity has been debated and suggests a potential new target for antithrombotic therapy (Woodruff et al., 2011).

2.2.1 Inflammation (kallikrein-kinin system)

Active FXII initiates the kallikrein-kinin system, which liberates the inflammatory mediator BK. FXIIa cleaves PK, yielding kallikrein, which in turn can cleave HK to release BK. The short-lived hormone BK acts through activation of G protein-coupled B_1-receptors or B_2-receptors. B_2-receptors have a high affinity for BK and are expressed in multiple tissues, while B_1-receptor expression is increased in pathophysiological events like tissue injury or inflammation. Through the activation of the receptors, BK initiates an increase in intracellular calcium, which leads to increased vascular leakage and pain sensation via prostacyclin and nitric oxide. Finally activation of the G protein-coupled BK-receptors dilates vessels and increases vascular permeability (Labberton et al., 2015).

Excessive activation of the complement- or contact system and BK-mediated edema formation is a consequence of a deficient/non-functional C1INH, or mutated FXII (Bjorkqvist et al., 2015). Hereditary angioedema is a rare inherited disease, characterized by acute swelling, abdominal pain and potentially life-threatening upper airway obstructions. Patients

with mutations in C1INH or FXII have excessive BK formation due to pathological activation of the kallikrein-kinin system (Bjorkqvist et al., 2013b).

2.2.2 Coagulation

Active FXII triggers fibrin formation by the intrinsic pathway of coagulation. A commonly used clinical test, the activated partial thromboplastin time (aPTT), is based on FXII activation by contact mediated activation. The aPTT measures the intrinsic pathway of coagulation by adding kaolin (a silicate) to plasma. The test is frequently used to monitor heparin treatment in patients. However, even if FXII is important for fibrin formation *in vitro*, FXII-deficient patients have a completely normal hemostasis and do not suffer from any bleeding complications, despite their prolonged aPTT (Ratnoff & Colopy, 1955). The missing bleeding complications in FXII-deficient patients is contradictory to most other coagulation factor deficiencies, like FVIII, FIX, FVII and vWF, where patients have spontaneous and recurrent bleeds (Labberton et al., 2015). As with FXII deficiency, people who lack the contact proteins HK or PK do not have obvious bleeding problems (Girolami et al., 2010). This results in the hypothesis that FXII-driven contact activation is not essential for fibrin formation *in vivo* and that hemostasis is initiated fundamentally through the extrinsic pathway of coagulation.

Since the first identified FXII deficient patient (Mr. Hageman) died of pulmonary embolism, the potential relation between FXII deficiency and venous thrombosis has been debated. However, when case reports of venous thrombosis in patients with FXII deficiency are evaluated, it is shown that in most cases FXII deficiency was associated with other inherited or acquired prothrombotic risk factors (Girolami et al., 2004). Thus, more than 60 years after the first description of FXII deficiency in humans (Ratnoff & Colopy, 1955) and after the discovery that FXI can be activated in an FXII-independent manner (Gailani & Broze, 1991) FXII has still no known function in hemostasis. Like humans, mice deficient in FXII do not have impaired hemostasis and reconstitution with human FXII normalized the prolonged aPTT (Pauer et al., 2004). Surprisingly FXII-deficient mice are protected in arterial and venous thrombosis models where reconstitution with FXII restored the defective thrombotic response (Renne et al., 2005). Since FXII plays a role in pathological thrombus formation and appears to be dispensable for hemostasis, targeting FXII, FXIIa or the activators of FXII can be a promising target for antithrombotic therapy with low or no risk of bleeding.

2.3 Activators of the contact system

Surface-dependent activation of FXII is caused by negatively charged substances, which induce a conformational change in FXII resulting in a structure that is more prone to proteolytic activation (Griffin, 1978). Many non-physiological activators of FXII are known, however the endogenous activator of the contact system and requirements for activators to induce contact activation are still not precisely known.

2.3.1 In vitro activators

Non-physiological surfaces such as glass, kaolin and ellagic acid are known to activate FXII and common coagulation assays, as aPTT, use this to auto-activate FXII on these negatively charged surface. The non-physiological polysaccharide dextran sulphate (DXS) is known as a contact system activator to induce BK mediated hypotension. DXS initiates only BK formation via activation of the kalikrein-kinin system but does not trigger fibrin formation (Siebeck et al., 1994). A problem shows up when blood is exposed to artificial non-physiological procoagulant surfaces used to treat cardiovascular diseases, such as vascular grafts, stents, and catheters. Subsequent thrombus formation on these devices is a common cause of failure (Jaffer et al., 2015). FXIIa levels are elevated in patients following cardiac stenting (Ponitz et al., 2009), in patients on hemodialysis (Frank et al., 2013) and patients who are on extracorporeal membrane oxygenation (Larsson et al., 2014).

2.3.2 In vivo activators

The discovery that deficiency in FXII results in normal bleeding let to the question what the natural FXII contact activators is *in vivo*. Until now various activators have been found that seem to activate FXII in different ways (**Figure 5**).

2.3.2.1 Nucleic acids

Nucleic acids can provide a surface, which allows binding and activation of the contact system. Extracellular RNA was recognized as a FXII activator and targeting the polymer with RNAse provided protection in thrombosis models (Kannemeier et al., 2007). Likewise, activates single-stranded DNA FXII *in vitro* (Pavlov et al., 2006) and neutrophil extracellular traps (NETs), consisting of DNA components can provide a surface for contact activation (Oehmcke et al., 2009). The capacity of NETs to trigger FXII activation significantly increased in the presence of activated platelets. This suggests that FXII can bind to NET surfaces and can be converted to FXIIa, which is relevant for propagation of deep vein thrombosis (von Bruhl et al., 2012).

2.3.2.2 Misfolded proteins

Misfolded protein aggregates are a type of activator, which leads to the activation of the kallikrein-kinin system, but not to the initiation of fibrin formation. Maas et al. found that FXII, but not FXI, is activated and that kallikrein formation occurs in blood from patients with systemic amyloidosis, a disease where misfolded proteins deposit in organ or tissues (Maas et al., 2008). However β-amyloid, a misfolded protein in Alzheimer's disease, is found to result in FXII-driven FXI activation and thrombin generation in human plasma, thus establishing misfolded proteins as a possible driver of thrombosis (Zamolodchikov et al., 2016).

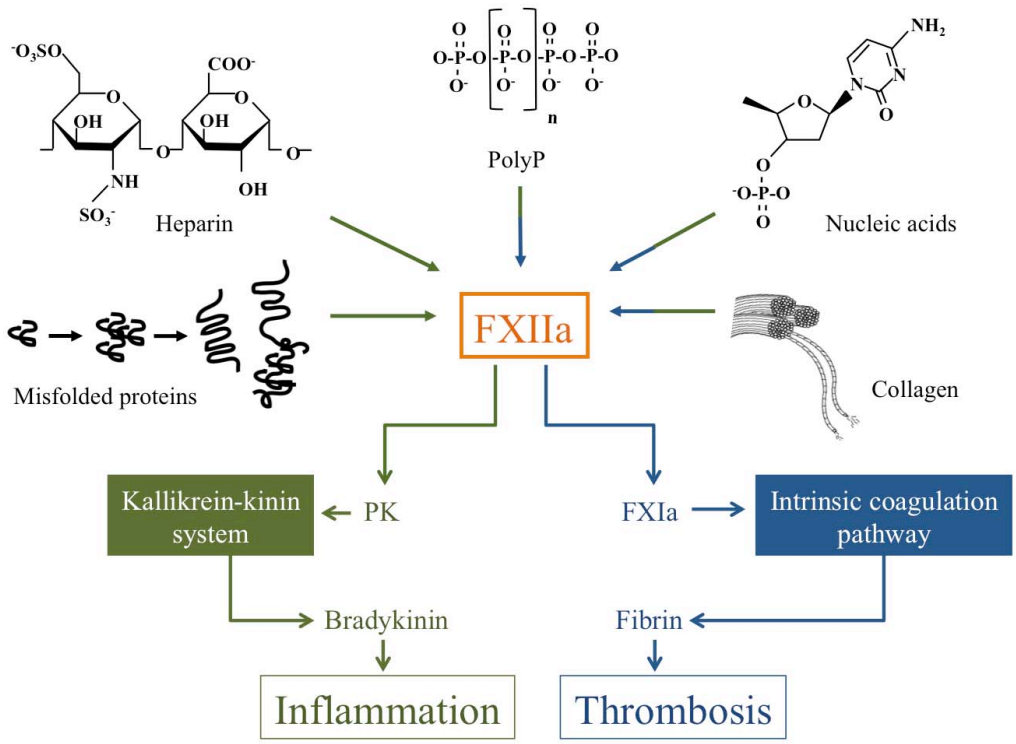

FIGURE 5: *In vivo* activators of the contact system.
FXII zymogen is *in vivo* activated upon binding to the natural FXII contact activators collagen, nucleic acids, polyphosphate (polyP), heparin and misfolded proteins. Where polyP, nucleic acids and collagen result in both activation of the kallikrein-kinin system and the intrinsic pathway of coagulation, heparin and misfolded proteins can only activate the kallikrein-kinin system. Modified with permission (Labberton et al., 2015).

2.3.2.3 Heparin

Heparin, a linear and highly sulfated polysaccharide, is found in mast cells and was discovered as an *in vivo* activator of the FXII-driven proinflammatory contact system, which contributes to swelling, anaphylaxis and inflammatory diseases. Deficiency in FXII or B_2-receptors protects mice from heparin and activated mast cell-induced edema formation (Oschatz et al., 2011). An anaphylactic shock, as a result from rapid release of mast cell-derived mediators into the blood stream is associated with increased heparin levels, activation of the contact system and BK formation. Sala-Cunill *et al.* showed that plasma samples from patients that were taken during the acute phase of anaphylaxis revealed activation of FXII and PK, while in basal conditions no contact system activation was detected (Sala-Cunill et al., 2015). Heparin, like DXS, specifically only triggered FXII-mediated BK formation and has no effect on FXI activation (Oschatz et al., 2011). As an antithrombin enhancer, commercial heparin is widely used as an anticoagulant drug. Heparin is highly heterogeneous and the structure of commercial heparin or heparin derived from human mast cells shows dissimilarities (Bianchini et al., 1997). Different heparins activate the contact system with different potency. In 2008 many lethal acute hypersensitivity reactions were noticed in patients who received commercial heparin (Blossom et al., 2008). A non-natural over-sulfated chondroitin sulfate (OSCS) contaminant was identified in this commercial available heparin (Guerrini et al., 2008). Potency for FXII activation is depended on negative charge

density and OSCS with an average of four sulfate residues per disaccharide is much more potent than mast cell heparin (average of 2.7 sulfate residues, Oschatz et al., 2011).

2.3.2.4 Collagen

Non-collagenous and collagenous proteins in the basement membrane contribute after injury or plaque rupture to the activation of the intrinsic pathway of coagulation. Vascular collagen can activate the intrinsic pathway of coagulation even in the absence of platelets, showing that collagen binds FXII and causes its activation (van der Meijden et al., 2009). Additionally a role for FXII-driven coagulation in arterial thrombosis on atherosclerotic plaques is suggesting a combined role of collagen, platelets, and fibrin in activation of the FXII pathway (Kuijpers et al., 2014b). Moreover, laminin the most abundant non-collagenous protein in the basement membrane, shortened the clotting time of plasma in a FXI- and FXII-dependent manner (White-Adams et al., 2010).

3. POLYPHOSPHATE

Recently, a new natural contact activator is found, polyphosphate (polyP, Muller et al., 2009). PolyP is an inorganic polymer that is found in every cell, yet its main functions has long been unknown. This mysterious cell component was considered as 'molecular fossil' and has been ignored in most biochemistry textbooks. Today, polyP is widely used as fertilizer, flame retardant, food additive and in water treatment because it is inexpensive, nontoxic and biodegradable. Additionally, polyP has numerous and varied biological functions in different organisms depending on where it is found (Kulakovskaya et al., 2012).

3.1 Chemical structure of polyP

The most structurally simple and the least characterized polymer in biological systems is polyP. PolyP is an inorganic polymer of tens to thousands of orthophosphate residues linked together by 'high-energy' phosphoanhydride bonds (**Figure 6**). Linking each phosphorus atom to its neighbor through an oxygen atom forms a linear and highly negative charged structure.

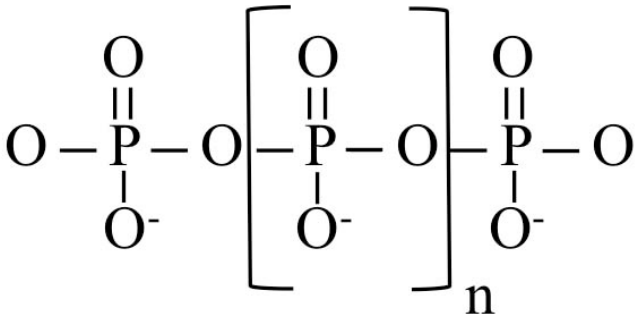

FIGURE 6: Structure of polyP.
A negative charged polymer of orthophosphate residues. The values for *n* [PO_3^-] residues can vary from tens to many thousands dependent on cellular localization.

Polyphosphates are salts that in solution contain hydroxyl groups, which are strongly acidic. They are complexing agents for many metal ions and the alkali metal salts of polyP are soluble in water. Though complex formation of polyP with the biological important cations Ca^{2+} and Mg^{2+} results in insoluble particle formation (Donovan et al., 2014). PolyP is present in all known living cells in both free and bound forms (Kulaev et al., 2004).

3.2 Biosynthesis of polyP

Certain enzymes related to the synthesis of polyP have been identified, however only a few of them are engaged in polyP synthesis in eukaryotes. The enzymes purified from prokaryotes are quite well characterized, but the basis of polyP formation in yeast and mammals is still unclear.

3.2.1 Prokarytes

The most studied enzyme in polyP synthesis is the enzyme polyphosphate kinase (PPK1) discovered in *E. coli*. PPK1 catalyzes the transfer of energy-rich phosphate residues from ATP to polyP, a reversible reaction. This membrane-bound enzyme synthesizes long chains of polyP *in vivo*, needs Mg^{2+} for its activity and is accountable for the biggest part of polyP metabolism in bacteria (Kulaev et al., 2004), seen by a significant decrease of polyP production in a PPK1 mutant of *E. coli* (Rao & Kornberg, 1996). However, in a mutant of *P. aeruginosa* deficient in PPK1, polyphosphate kinase activity was still noticed and this lead to the discovery of PPK2 (Ishige et al., 2002). PPK2 favors synthesis of polyP from GTP and GDP and prefers Mn^{2+} to Mg^{2+}. Because polyP deficiency results in incomplete growth in the stationary phase of the bacteria, polyphosphate kinases have been suggested as one of the targets of novel antimicrobial drugs (Rao et al., 2009).

3.2.2 Eukaryotes

Where PPK1 is highly conserved among all bacteria and archaea, currently only one eukaryotic organism (a slime mold) is known to possess a PPK1 homolog. Besides this, one other eukaryotic enzyme involved in polyP synthesis has been identified and characterized from the budding yeast *Saccharomyces cerevisiae*. This enzyme, vacuolar transporter chaperone (VTC), has been shown to possess polyP polymerase activity by using ATP to generate polyP and also participates in polyP transport across membranes. Mutations in VTC block polyP translocation and synthesis resulting in undetectable polyP accumulation in the yeast vacuoles (Cohen et al., 1999). Reconstitution of polyP translocation with purified vacuoles results in production of toxic levels of polyP in the cytosol of yeast, connecting synthesis of polyP by VTC directly to transport of polyP to avoid accumulation of polyP in the cytosol (Gerasimaite et al., 2014).

Moreover, it is shown that yeast polyP levels are metabolically linked to the presence of a family of high-energy phosphate-rich molecules called inositol pyrophosphates. Synthesis of inositol hexakisphosphate by IP6 kinase 1 (*IP6K1*) seems to be critical in this process and yeast lacking *IP6K1* have undetectable levels of inositol pyrophosphates and reduced levels

of polyP (Auesukaree et al., 2005). The enzymes responsible for polyP synthesis in mammals are still not known, though mammals have isoforms of *IP6K1*. Interestingly a mouse deficient in *IP6K1* displays significant lower polyP levels in platelets showing that the link between inositol pyrophosphate and polyP levels is conserved in mammals (Ghosh et al., 2013). Despite this link the main pathway of polyP synthesis in mammalians is still in question.

3.3 Degradation of polyP

PolyP can be degraded by different endo- and exopolyphosphatases. The enzymes differ between prokaryotes and eukaryotes and even belong to different protein families. Moreover many degradation enzymes are multifunctional and can catalyze reactions both with polyP and nucleoside phosphates (Kornberg, 1995).

3.3.1 Prokaryotes

The discovery of polyphosphate-glucose phosphotransferase in bacteria provided the first evidence of an energy donor besides the normally used nucleoside phosphates. The enzyme can use polyP as phosphoryl donor to phosphorylate glucose. This suggests that polyP could have been the precursor of ATP in the beginning of evolution and that there has been a conversion from polyP to ATP as the phosphoryl donor (Kulaev et al., 2004).

The most important enzymes involved in polyP degradation in prokaryotes and yeast are exopolyphosphatases, enzymes that hydrolyze the terminal phosphate anhydride bonds of a polyP chain. Two main exopolyphosphatases are found in bacteria. They both possess one hydrophobic region for substrate binding and belong to the same sugar kinase/actin/heat-shock protein hsp70 superfamily (Keasling et al., 1993, Reizer et al., 1993). These two enzymes in *E. coli* are exopolyphosphatase (PPX) and guanosine pentaphosphate phosphohydrolase (GPPA).

The cytoplasmic phosphatase PPX is a dimer where one unit is 513 amino acids with a molecular mass of 58 kDa. It is a highly processive enzyme especially for polyP of long chain lengths (longer than fifteen phosphate units) and it fails to act on ATP and ADP. PPX and PPK1 are found together in one operon, which implies a co-regulation between these two proteins (Akiyama et al., 1993). PPX is composed of four distinct domains and multiple polyP binding sites are recognized to be responsible for the recognition of only long-chain polyP (**Figure 7**). The N-terminal domains 1 and 2 harbor the enzymatic cleft, whereas the C-terminal domains 3 and 4 are responsible for nearly all affinity for polyP (Bolesch & Keasling, 2000, Rangarajan et al., 2006).

GPPA is a bi-functional enzyme, which besides exopolyphosphatase activity regulates the conversion of guanosine pentaphosphate (pppGpp) to the effective second messenger guanosine tetraphosphate (ppGpp). ppGpp is also known as the magic spot in the ‘stringent response’, the response of *E.coli* on the regulation of cell metabolism when the amount of essential nutrients suddenly drops (Kristensen et al., 2004). Increased levels of ppGpp inhibit PPX activity meaning that in response to nutrient stress, bacteria accumulate polyP.

Subsequently, binding of polyP with particular ribosomal proteins can activate proteases and control degradation of free ribosomal proteins. Therefore, by directly affecting PPX activity, bacteria can recapture amino acids from protein synthesis, inhibit expression of ribosomal genes and prevent starvation (Dalebroux & Swanson, 2012).

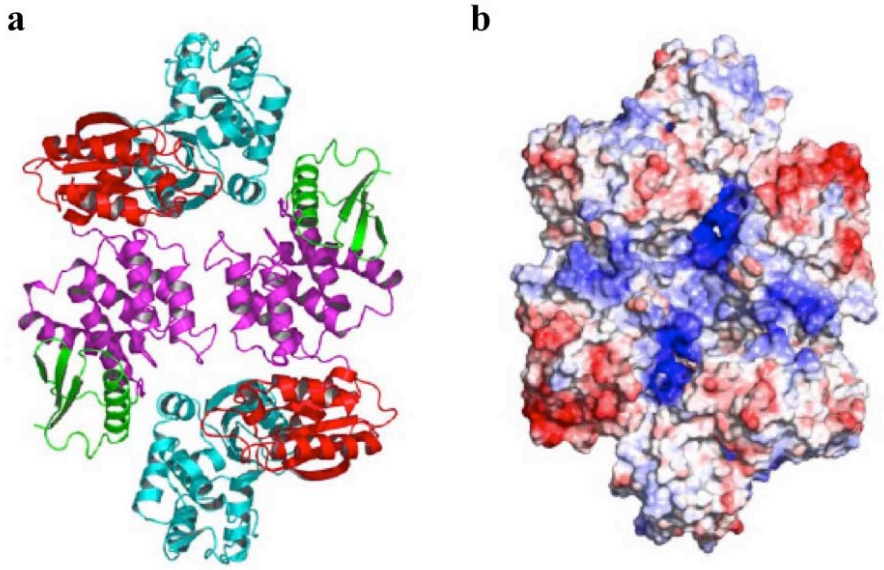

FIGURE 7: Structure of the *E. coli* PPX dimer and the putative active site canyon.
(a) Ribbon representation of the PPX dimer, domains I (red), domains II (cyan), domains III (magenta) and domains IV (green) are shown. (**b**) Electrostatic potential mapped to the molecular surface representation of the dimer, showing regions of positive potential within the putative polyP-binding cleft. Modified with permission (Rangarajan et al., 2006).

3.3.2 Eukaryotes

Exopolyphosphatases of the yeast *Saccharomyces cerevisiae* was the first purified exopolyphosphatases with high activity and stability. It is found in the cell envelope, cytosol, vacuole and in mitochondrial matrixes. The comparison between the different exopolyphosphatases in yeast suggests that they are compartment-specific enzymes and that they vary in kinetic properties and substrate specificity (Wurst & Kornberg, 1994). The shared inhibitor for all exopolyphosphatases is heparin (Lichko et al., 2000). Exopolyphosphatase activity is also found in mammalian cells and tissues, in synovial fluid as well as in human blood (Leyhausen et al., 1998). Several enzymes, such as alkaline phosphatase, have been suggested as polyP degrading enzyme in mammals (Lorenz & Schroder, 2001), however no specific exopolyphosphatase has yet been found.

Besides exopolyphosphatases, endopolyphosphatases are identified, which hydrolyze internal phosphate anhydride bonds leading to products containing four to five phosphate residues. Endopolyphosphatases have been purified from yeast and mammals, though have not been seen in prokaryotes (Kumble & Kornberg, 1996).

3.4 Localization and forms of polyP

PolyP was reported already in 1888 when it was found in nuclei of yeast (Lieberman, 1888). Subsequent data shown that polyP is found in all living organisms at different stages of evolution. Being a polyanion gives polyP the ability to form multiple complexes with many different biologically components.

3.4.1 Prokaryotes

Prokaryotic cells have a simple cell structure, and polyP is found in all main compartments. PolyP is located in the so-called metachromatic granules (volutin granules), which appear red when stained with methylene blue. Complexes of polyP with heavy metals like Sr^{2+}, Ba^{2+}, Ni^{2+} and Cd^{2+} are found and bacterial membranes contain polyP in complex with Ca^{2+} and polyhydroxybutyrate to form membrane channels (Reusch, 2000).

3.4.2 Eukaryotes

Eukaryotic cells are more developed than prokaryotic cells and are know to have specialized organelles. The data so far, show that all cell compartments of eukaryotes seem to contain pools of polyP. In eukaryotes, localization of polyP has mostly been studied in yeast where polyP, similarly to bacteria, is present in volutin granules and in complex with arginine, lysine, Mg^{2+}, Ca^{2+} and Mn^{2+}(Kulaev et al., 2004). Unicellular eukaryotic organisms and some algae contain pyrophosphate and polyP storage organelles called acidocalcisomes, which are similar to the volutin granules found in yeast. Acidocalcisomes are acidic electron-dense granules, which have polyP in complex with different cations like Ca^{2+}, Mg^{2+} and Zn^{2+} (Docampo et al., 2010).

In mammalian cells polyP is present in organelles similar to acidocalcisomes such as platelet dense granules (Ruiz et al., 2004) and granules of mast cells and basophils (Moreno-Sanchez et al., 2012). Indicating that granules that are full of polyP, calcium and other cations are preserved from bacteria to human. PolyP is also found in the lysosomes of human fibroblasts (Pisoni & Lindley, 1992), in osteoblasts (Leyhausen et al., 1998), on cancer cells (Jimenez-Nunez et al., 2012) and on cancer cell-derived membrane vesicles (Nickel et al., 2015). Membrane channels of complexes between Ca^{2+}, polyP and polyhydroxybutyrate were also identified in eukaryotic cells especially in mitochondria (Pavlov et al., 2005) and variable polyP concentrations have also been measured in heart, brain, liver and kidney cells (Kumble & Kornberg, 1995).

3.5 Biological functions of polyP

PolyP performs numerous functions in living cells and this has been mainly studied in prokaryotes. PolyP is abundant in prokaryotes and lower eukaryotes and seems to be essential for growth of cells, the response to stress, gene control and regulation of enzymes. Higher eukaryotes have smaller amounts of polyP per cell and the main function of this polyanion is probably more related to participation in regulatory processes (Kornberg et al., 1999).

3.5.1 Phosphate storage and energy source

PolyP is a regulator of the intracellular level of phosphate in prokaryotes. Constant levels of free monophosphate are necessary and significant increased levels of monophosphate will change the osmotic pressure and pH in a cell. PolyP is a stable form of phosphate reserve, which can be build up or broken down in periods of phosphate excess and starvation, respectively. PPK1 can also convert polyP to ATP and polyP qualifies as energy equivalence to ATP (Kulaev et al., 2004).

3.5.2 Chelator of metal ions and complex formation

As a polyanion, polyP strongly chelates metal ions. The regulation of cellular Ca^{2+} and Mg^{2+} by polyP is fundamental in the cell walls of bacteria, providing a possible mechanism for the antibacterial capacity of polyP (Lee et al., 1994). Furthermore is polyP involved in the detoxification of heavy metal cations (e.g., Ni, Zn, Fe, Cu, and Cd, Kornberg, 1995). PolyP as membrane channel in complex with Ca^{2+} and polyhydroxybutyrate was discovered in the membranes of competent cells. The complex induced changes in the cell membrane, possibly facilitating DNA entry into these cells. Both in prokaryotes and eukaryotes participates polyP by forming a complex in multiple membrane transporters (Reusch, 2000).

3.5.3 Regulator for stress and survival

A regulatory role for polyP seems reasonable, since polyP is structurally comparable to the nucleic acids RNA and DNA. PolyP can interact with basic proteins or basic domains (histones, polymerases) and in this way affect gene regulation. Accumulation of polyP is seen in the nucleolus of myeloma cells to modulate transcription (Jimenez-Nunez et al., 2012). The involvement of polyP in the 'stringent response' in which many genes are induced shows that polyP has regulatory influences to cope with environmental stress and to ensure survival (Dalebroux & Swanson, 2012). PolyP can also regulate stress in bacteria by acting as a chaperone where it stabilizes proteins in stress conditions. It reduces the need for other chaperone systems and protects different proteins against stress-induced folding and aggregation by binding to them in ATP-independent way (Gray et al., 2014).

3.5.4 Other functions of polyP

PolyP can up-regulate inflammatory pathways (Bae et al., 2012), regulates cell growth by activation of mTOR, a kinase involved in the proliferation of breast cancer cells (Hassanian et al., 2015) and enhances the proliferation of fibroblasts (Shiba et al., 2003). Moreover, polyP is involved in the modulation of the mineralization procedure in bone tissues (Schroder et al., 2000). *In vitro* and *in vivo* studies show that polyP dampens complement activation via the terminal pathway by destabilizing complement complex C5b-6 (Wat et al., 2014) and via the classical pathway by binding to both complement subcomponent C1 and C1INH potentiating the inhibitory function of C1INH (Wijeyewickrema et al., 2016). Another indication that polyP plays a role in innate immunity is by enhancing the platelet factor 4 mediated bacterial host defense mechanism (Brandt et al., 2015).

3.6 PolyP as procoagulant polymer

It was shown already in 1965 that platelets initiate FXII-driven coagulation (Castaldi et al., 1965). Other studies including whole blood assays and multiple studies in plasma showed that activated platelets promote coagulation in a FXII-dependent manner (Back et al., 2010, Johne et al., 2006, Nielsen et al., 2005, Walsh, 1972, Walsh & Griffin, 1981). In 2004 Ruiz *et al.* found that platelet dense granules contain polyP (Ruiz et al., 2004) and in 2006 it was discovered that synthetic polyP can activate FXII (Smith et al., 2006). Putting these findings together Muller *et al.* showed that polyP-driven FXII activation is a necessary mechanism in platelet-driven thrombosis *in vivo* (Muller et al., 2009). Various other groups have confirmed that polyP released from platelet dense granules initiates clotting via the contact pathway by acting as an anionic surface to trigger FXII cleavage (Hernandez-Ruiz et al., 2009, Puy et al., 2013, Smith et al., 2010). Furthermore plasma clotting times of hemophilia patients or patients receiving the anticoagulant warfarin, can be normalized by addition of polyP (Smith & Morrissey, 2008a). Additionally, polyP reversed the defect in vWF-dependent platelet agglutination in type I von Willebrand disease patients (Montilla et al., 2012). Finally, prolonged bleeding times are found in patients with a defective biogenesis of platelet dense granules (Hermansky-Pudlak syndrome, Gahl et al., 1998). These patients have substantially lowered levels of polyP in their platelets compared with the general population (Hernandez-Ruiz et al., 2009) and delayed *in vitro* clot formation in such patient samples was shortened on addition of polyP (Muller et al., 2009).

Besides initiating fibrin production by activating FXII, polyP has shown *in vitro* to have additional procoagulant functions (**Figure 8**). PolyP amplifies fibrin production by accelerating the feedback-activation of FXI by thrombin (Choi et al., 2011) and the conversion of factor V to Va by FXa, thrombin and FXIa (Choi et al., 2014, Smith et al., 2006). The rapid formation of factor Va antagonizes the anticoagulant activity of TFPI and causes a localized thrombin burst, which slows fibrinolysis by augmenting the activity of TAFI (Smith et al., 2010). In addition, integration of polyP into fibrin clots causes thicker fibrin fibers and a change in fibrin distribution, resulting in slower fibrinolysis because of interference with the binding of tissues plasminogen activator and plasminogen to fibrin (Smith et al., 2006, Mutch et al., 2010).

Plasma experiments suggested that the chain length of the polymer regulates the relative potency of polyP in activating the various pathways. In supernatant of activated platelets polyP with a chain length of 60-100 phosphate units is found, and this length accelerates blood-clotting reactions but was less efficient in initiation of coagulation. Instead, microorganism polyP molecules, which are hundreds to thousands of phosphate units long were the optimal activators of the contact pathway (Smith et al., 2010).

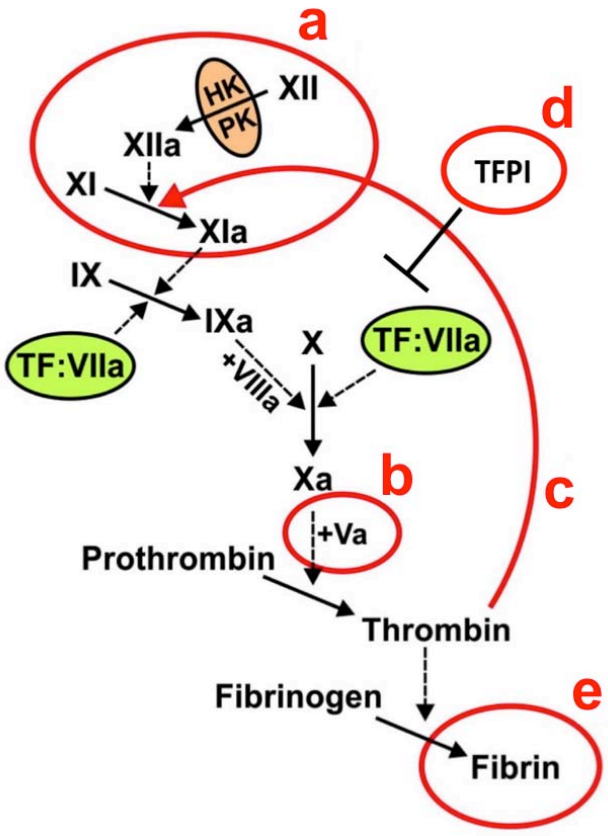

FIGURE 8: Different roles of polyP in coagulation.
It is shown *in vitro* that synthetic polyP can have different procoagulant effects. PolyP can initiate fibrin formation by activating the intrinsic pathway of coagulation (**a**) and amplify thrombin formation by accelerating factor V activation (**b**) and factor XI feedback-activation (**c**) plus abrogating TFPI function (**d**). Additionally enhances polyP fibrin polymerization and slows down fibrinolysis (**e**). Modified with permission (Morrissey, 2012).

3.7 Analysis of polyP

Because of its simple structure, researchers working with polyP face several difficulties in analyzing the polymer. There is a lack of sensitive techniques for manipulating and detecting polyP at its biological concentration. Since polyP is unstable in plasma and has a simple, repetitive structure, production of anti-polyP antibodies has not been successful to date. PolyP can be detected in polyacrylamide gels by using metachromatic staining by toluidine blue or 4',6 diamidino-2-phenylindole (DAPI) staining (Smith & Morrissey, 2007). The same staining techniques are used to stain, detect, and to some extent to quantify polyP in solutions as well as in cells or tissues. Currently, quantification of polyP can only be performed by complete acid hydrolysis to monophosphate and colorimetric analysis using a malachite green assay (Morrissey et al., 2012). Due to the recent recognition of the inorganic polymer polyP as a procoagulant platelet-derived inflammatory mediator, improvements in assay methods for use in plasma and tissues are needed. Several studies have corroborated a central role of the polymer for thrombosis, giving a potential role for platelet polyP as a new biomarker. Establishing an assay to monitor polyP in patients samples constitutes a novel method that can potentially be employed in the early diagnosis and treatment monitoring of thromboembolic diseases.

4. PREVENTION AND TREATMENT OF THROMBOSIS

Thrombosis is a common, lethal disorder and if not deadly will reoccur frequently and result in long-term complications. These facts and the associated economic burden underline the need for effective prevention of thrombosis. Understanding the processes that results in thrombosis is crucial for developing more effective and saver antithrombotic drugs.

4.1 Currently used anticoagulants

In general, arterial thrombosis is treated with platelet targeting drugs, where the treatment of venous thrombosis consists of drugs that target coagulation. In patients with cardiovascular diseases the available antithrombotic drugs are effective, however, the primary complication of all currently used anticoagulant drugs are significant bleeding risks (Mackman, 2008).

4.1.1 Arterial thrombosis

The main targets of drugs used to treat arterial thrombosis are platelet activation and platelet aggregation. Therefore, antiplatelet drugs are the first choice for prevention and treatment of arterial thrombosis. The most commonly used drug, aspirin, interferes with thromboxane A_2 activation of platelets by inhibiting cyclooxygenase 1. Aspirin is shown to significantly reduce the risk of a combination of cardiovascular events and moreover reduces the risks in patients who previously have had a myocardial infarction. Nevertheless, it significantly increases the risk of bleeding and can cause stomach ulcers (Berger et al., 2006). Another antiplatelet drug is clopidogrel, which is used to treat patients with coronary syndromes and reduces platelet activation and aggregation. Similarly to aspirin, treatment results in marked prolongation of the bleeding time in patients (Gachet, 2005).

4.1.2 Venous thrombosis

In the treatment of venous thrombosis, drugs targeting proteins of the coagulation cascade are used. These drugs prevent growth and embolization of the thrombus. A patient with venous thrombosis will first receive heparin, which acts quickly. Heparin increases the function of the natural anticoagulant antithrombin to inhibit FXa and thrombin. Vitamin K antagonists are used for long-term, preventative anticoagulant therapy. Vitamin K is needed for post-translational modification of several coagulation factors (factor IX, factor X, factor VII and prothrombin). Vitamin K antagonists inhibit the enzyme vitamin K epoxide reductase resulting in an interruption of the vitamin K cycle and a lack of vitamin K for the production of coagulation factors in the liver. Both heparin and vitamin K antagonist have been used for over 50 years. Lately, new oral anticoagulants are on the market, like rivaroxaban and dabigatran, which selectively inhibit FXa and thrombin, respectively. Nevertheless, similar to heparin and vitamin K antagonists the new oral anticoagulants still have significant bleeding risks as side effect (Baber et al., 2015).

4.2 Targeting factor XII(a)

The findings that FXII deficient patients have normal bleedings and FXII-deficient mice are protected in thrombosis models, resulted in the possibility of antithrombotic drugs that target FXII or FXIIa without significantly affecting hemostasis. The first known inhibitor of FXIIa was corn trypsin inhibitor (CTI). CTI reversibly and selectively interacts with the active site of FXIIa. It is effective when added to plasma in prolonging the aPTT (Hojima et al., 1980) and because of its small size, CTI is suitable for immobilization on catheter surfaces for the prevention of catheter-initiated clotting. CTI-coated catheters attenuated FXIIa-mediated activation of FXI in rabbits resulting in prolonged time to catheter occlusion (Yau et al., 2012). Another biological derived FXIIa inhibitor is Infestin 4, a serine protease inhibitor derived from the gut of the kissing bug (Triatoma infestans, Campos et al., 2004). Hagedorn *et al*. fused Infestin 4 to recombinant human albumin to enhance the bioavailability and challenged mice and rats in pathological thrombus formation models (Hagedorn et al., 2010). Profound protection from thrombosis was obtained. Recently, a humanized antibody (3F7) was developed that targets specifically the active site of FXIIa. This antibody interfered with FXIIa-mediated coagulation and blocked experimental thrombosis in mice and rabbits. Additionally, 3F7 provided thromboprotection in an extracorporeal membrane oxygenation cardiopulmonary bypass system in rabbits. 3F7 treatment did not increase bleeding from wounds, unlike heparin, nevertheless thrombroprotection was as efficient as heparin (Larsson et al., 2014).

By inhibition of the zymogen FXII with antibody 15H8, Matanfonov *et al.* showed that the prothrombotic effect of FXII was completely blocked in an arterial mouse model. Moreover fibrin and platelet accumulation downstream of an inserted graft was reduced in 15H8 treated primates supporting the role for FXII in thrombus formation (Matafonov et al., 2014). Antisense oligonucleotides (ASO) used to selective knockdown FXII attenuated catheter induced thrombosis in rabbits (Yau et al., 2014). Additionally, ASO treatment in mice reduced thrombus formation without an effect on hemostasis (Revenko et al., 2011). Surprisingly, reduced FXII levels by small interfering RNAs (siRNA) propagated development of spontaneous venous thrombosis in mice (Heestermans et al., 2016). By using siRNA only a reduction of 86% in mRNA levels (*vs.* 96% reduction by ASO) was seen, resulting in a plasma protein activity of FXII about 20% and normal thrombin generation and aPTT values (Heestermans et al., 2016). This shows that plasma FXII levels have to be close to zero to have an antithrombotic effect. Because FXII zymogen is in much higher concentrations available in plasma than the active protease, targeting FXIIa is considered to be a better strategy than targeting FXII. However, high concentrations of Infestin-4 and CTI have off-target effects *in vivo* on FXa and FXIa, respectively, which is a limitation of these molecules (Hansson et al., 2014, Xu et al., 2014).

4.3 Targeting polyP

As shown above, targeting FXII or FXIIa interferes with thrombosis without significantly affecting hemostasis. Blocking the natural activator of FXII, the procoagulant polymer polyP

is an appealing novel antithrombotic strategy. The earlier mentioned knockout mice in *IP6K1*, which displays significant lower platelet polyP levels, support this theory. *IP6K1* deficiency results in protection against pulmonary thromboembolism indicating a role for polyP in thrombosis (Ghosh et al., 2013).

Since the finding that polyP activates FXII, some attempts have been made to target polyP. Treatment of polyP with alkaline phosphatase (a polyP degrading enzyme) abrogated activation of the contact system and blocked platelet-induced thrombosis in mice (Muller et al., 2009). However alkaline phosphatase is a nonspecific enzyme that can degrade other phosphate-containing compounds such as ADP and ATP (Akiyama et al., 1993). That a variety of peptides and small molecules from the saliva of blood-feeding animals can inhibit the hemostatic and inflammatory systems is already shown with the FXIIa inhibitor infestin-4 (Hagedorn et al., 2010). PdSP15a and b are proteins that are found in the saliva of the African sand fly. Alvarenga *et al.* showed that PdSP15a and b bind the polymers DXS, heparin and polyP and act as effective inhibitors of the contact activation of FXII, though they also interfere with silica-driven plasma clotting possibly because of their binding to other polymers (Alvarenga et al., 2013).

Furthermore, polyamidoamine dendrimers (PAMAM) bind with high affinity to prothrombotic nucleic acids and polyP, which results in strong antithrombotic effects in both carotid artery and pulmonary embolism thrombosis models without and increase in bleeding (Jain et al., 2012, Smith et al., 2012). Though PAMAM were not engineered to be antithrombotic agents and while attenuating thrombosis, they give significant toxicity *in vivo*. PAMAM contain multiple primary amines, which allows them to bind to and inhibit polyP, but this property also leads to cellular toxicity, platelet activation, and coagulopathy mediated by fibrinogen aggregation because of binding to proteins and cell surfaces (Jain et al., 2010, Jones et al., 2012). Travers *et al.* examined universal heparin reversal agents (UHRAs) and their ability to bind to polyP and inhibit its role in thrombus formation *in vivo* (Travers et al., 2014). UHRAs were designed by assembling multifunctional cationic groups into the core of a dendritic polymer, to protect them from non-specific interactions with blood components reducing toxicity *in vivo*. Intravenous injection of UHRAs in mice was well tolerated, they had a high affinity for polyP and interfered with *in vivo* thrombosis. However, even though UHRAs cause less bleeding than unfractionated heparin they still increase bleeding times approximately three times compared to controls. Furthermore, UHRAs interfered with TF-initiated fibrin formation (Travers et al., 2014).

Collectively, the present knowledge of the role of platelet polyP in hemostasis and thrombosis makes targeting polyP a possible novel strategy to interfere with thrombosis. Though, a selective non-toxic polyP targeting strategy has not exactly been found yet.

AIMS

The overall aim of this thesis was to provide insights into the mechanisms and regulation of the polyP/factor XII-driven contact system in thrombosis and hemostasis with a focus on inhibition of polyP. More specifically, the aims were:

I. Investigate localization of polyP on procoagulant platelets.

II. Target procoagulant polyP *in vivo*.

III. Analyse polyP on cells and in human samples.

EXPERIMENTAL PROCEDURES

The studies included in this thesis are based on a combined use of different *in vitro* and *in vivo* methodology adapted to the specific research questions. For a more detailed description of the methodology used, see the individual papers.

1. *IN VITRO* METHODOLOGY

Hemostasis depends on interactions between the plasma-based coagulation cascade, platelets and the endothelium, yet only the first two components can be tested in a test tube. Still laboratory measurements of coagulation represent a close approximation of the body's hemostatic system and they are important in nowadays practice.

1.1 Cloning, expression and purification of PPX deletion mutants (paper II, III and IV)

PPX was cloned from genomic DNA of *E. coli*, which was extracted with phenol-chloroform. Restriction sites were introduced into PPX and used to insert the amplified DNA fragment into the pTrcHisB expression vector, adding a N-terminal 6xHis-tag. Mutants based on the PPX domain-organization were cloned by PCR-based mutagenesis using pTrcHisB-PPX as template. Ultracompetent *E.coli* were transformed with pTrcHisB vectors coding the various PPX deletion mutants and recombinant protein expression in bacteria was induced with IPTG (isopropyl β-D-1-thiogalactopyranoside). Bacteria were harvested, lysed and the mutants were purified by affinity chromatography using the 6xHis-tag. Protein concentrations were determined by the Bradford method. Coomassie brilliant blue staining on SDS-PAGE and western blotting using a 6xHis-tag antibody assessed protein purity.

1.2 Enzyme-linked immunosorbent assay (ELISA) for polyP binding and detection (paper II and IV)

To analyze binding of PPX deletion mutants to polyP and to examine polyP on prostasomes, two ELISAs were developed. An ELISA involves detection of an antibody or an antigen in a sample, however since polyP has a simple structure, specific antibodies to capture polyP do not exist. By using EDAC (1-ethyl-3-[3-dimethylamino-propyl] carbodiimide)-mediated covalent coupling we immobilized polyP onto high-binding polystyrene 96-well plates (Choi et al., 2010). Immobilized polyP was incubated with the different PPX mutants and the bound proteins were quantified with a monoclonal antibody against 6xHis-tag, a HRP-coupled anti-mouse detection antibody and TMB substrate reaction. We identified the best polyP-binding mutant (PPX_Δ12, paper II), which we used for the second ELISA to examine polyP on prostasomes (paper IV). Prostasomes were immobilized to a 96-well plate and polyP on their surface was detected by using PPX_Δ12, 6xHis-tag antibody, HRP-coupled antibody and TMB substrate reaction.

1.3 PolyP characterization and staining (paper I, II and IV)

We resolved polyP, from different sources, on polyacrylamide TBE-urea gels, one of the most effective and widely used methods for polyP separation. Where mentioned samples were incubated with different concentrations of PPX or alkaline phosphatase for different time points. PolyP was visualized with DAPI-negative staining (Smith & Morrissey, 2007) and polyP samples with defined chain length were used as size standards. For the detection of polyP on prostasomes with fluorescence microscopy (paper IV), PPX_Δ12 was covalently coupled to amine-reactive Alexa594 to generate a fluorescently labeled polyP probe.

1.4 Platelet aggregation and recalcification time (paper II and IV)

Platelet aggregation measures the ability of platelet agonists to induce *in vitro* activation and platelet-to-platelet activation. Classical Born aggregometry is based on measurements of light transmission through platelet rich plasma (PRP, Born, 1962). PRP is initially turbid but allows for increasing transmittance of light when platelet aggregates form following platelet stimulation. Here we used ADP as platelet activator, which was pre-incubated with increasing concentrations of PPX. Recalcification time is normally a measurement to monitor the endogenous clotting process. Calcium is added to citrated whole blood and the time until clot formation is measured using a Kugel-koagulometer. Here we measured the recalcification time of human or murine PRP pre-incubated with trap6, collagen, and Ca^{2+} ionophore in the absence or presence of PPX, PPX_Δ12 or FXIIa inhibitory antibody.

Platelets are very sensitive and can be readily activated during the preparation of PRP. By drawing the blood without vacuum, discarding the first 10 ml of sample and processing the blood fast, careful and at room temperature we tried to avoid platelet activation. Additionally, we always included control PRP to which no platelet activators were added, these control samples underwent the same treatment as the samples with platelet activator.

1.5 Real-time thrombin generation (paper II and IV)

The measurement of real-time thrombin generation *in vitro* represents a relevant part of the *in vivo* system with all the plasma proteins present near their physiological concentrations, however, the vessel wall is lacking. Normally, to imitate vessel wall damage (e.g. TF) different triggers can be used. Though, our purpose is to measure FXII-mediated coagulation where addition of TF will blunt the procoagulant contact activation (Nickel et al., 2013). Therefore we analyzed thrombin generation with different contact activators in platelet poor plasma (PPP) without TF, containing phospholipids. Phospholipids are necessary to amplify the thrombin generation by providing a surface for the coagulation factors. Thrombin generation in PRP reflects the interplay between platelet activation and plasma coagulation. When using PRP no phospholipid addition is needed because the platelets take the role as amplifying surface. The most relevant results of thrombin generation are the lag-time, the time to peak, the peak height, and the endogenous thrombin potential (ETP), which are calculated by the analysis software (Hemker et al., 2006). Thrombin formation was induced

in PPP with different activators pre-incubated with PPX or PPX_Δ12 and in PPX- or PPX_Δ12 supplemented PRP upon stimulation with a platelet activator.

1.6 Amidolytic activity assays for FXIIa and FXIa (paper I, II and IV)

Formed FXIIa and FXIa in plasma by polyP or other activators were analyzed using the chromogenic substrates S-2302 and S-2366, respectively. Although these chromogenic substrates are commonly used to determine FXIIa and FXIa activity, other enzymes present in plasma can give conversion of the substrate (Tans et al., 1987). To overcome this limitation, we analyzed substrate cleavage in plasma after addition of the inhibitors of plasma kallikrein, thrombin, FXIa, FXIIa, FXa and thrombin.

1.7 Thrombus formation under flow (paper II)

A common limitation of *in vitro* coagulation assays is that they do not study circulating blood and do not take shear stress into account. Flow chambers are excellent tools to analyze the intrinsic pathway of coagulation where shear stress can be varied to simulate venous or arterial flow conditions (van der Meijden et al., 2009). Since collagen is exposed from the subendothelial matrix at sites of vascular injury we studied platelet driven contact activation on collagen-coated surfaces under flow. Flow chambers were co-infused with citrate-anticoagulated blood of human or mice and isotonic $CaCl_2$/$MgCl_2$ solution, which resulted in physiological Ca^{2+} and Mg^{2+} concentrations. Indicated blood samples were pre-incubated with increasing concentrations of PPX or PPX_Δ12 to determine their effect on thrombin formation in whole blood under flow.

1.8 PolyP analysis in platelet supernatants (paper II)

We evaluated the released polyP measured in monophosphate concentrations. The supernatant from activated platelets was collected and incubated with buffer or PPX. PPX specifically degrades polyP but not platelet-released ADP or ATP and this has been established for quantification of polyP (Ruiz et al., 2001). A malachite green-based phosphate assay kit, determined monophosphate in the supernatants. The difference of monophosphate in PPX- versus buffer-treated supernatant indicated released polyP. As a control for polyP not released from activated platelets, non-activated platelets were included in the same measurements.

1.9 Flow cytometry (paper III)

Flow cytometry analysis is based on the differences in the light-scattering properties and on fluorescent signals of single cells. It is a standard method to analyze platelet surface markers such as the integrin α2bβ3 and the glycoprotein complex Ibα/β-IX-V (van Velzen et al., 2012). To assess whether PPX_Δ12 has the capacity to probe for polyP on activated platelets, purified PPX_Δ12 was covalently coupled to amine-reactive Alexa488 to generate a fluorescently labeled polyP probe (PPX_Δ12-Alexa488). Platelet activation was measured as CD62P expression on the platelet surface. The mean intensity of the fluorescence (MFI) was analyzed for 10 000 platelets, gated by using their CD42b signal. The MFI value shows the

mean fluorescence signal from all individual platelets, and correlates to the number of fluorescent PPX_Δ12 that have bound to the polyP on the platelet surface. Since we used the same number of platelets, stained with the same concentrations PPX_Δ12-Alexa488 and used unstained controls we can compare the MFI between non-activated and activated platelets. We examined non-activated platelets incubated with increasing concentrations of synthetic polyP and platelets activated by different concentrations and type of platelet activators.

1.10 Platelet polyP extraction (paper I)

PolyP is commonly isolated from cells by using a phenol-chloroform extraction method (Kumble & Kornberg, 1995). This extraction method is selective for soluble polyP while water-insoluble, e.g., long-chain, aggregated or cell-bound polyP is discharged during the isolation procedure by removal of all cellular and cell-bound materials. Using the phenol-chloroform extraction method, we purified small quantities of short-chain (60-100 monomers) polyP from a large amount of activated platelets (Muller et al., 2009, Smith et al., 2010). By using an anion exchanger method (Werner et al., 2007) we purified polyP from whole platelet lysates, including membrane-associated and long-chain polyP.

1.11 Statistical methods

Sample sizes were determined empirically; no statistical tests were used to predetermine the size of the experiments. To check if data were normally distributed, a quantile-quantile plot was used and data were analyzed by Student's t test or, in the case of multiple comparisons, one-way analysis of variance (ANOVA) followed by post hoc analysis using Tukey's multiple comparisons test. Prism 6.0 (Graph Pad) was used for analysis, and the values of probability $p<0.05$ were considered as statistically significant.

2. *IN VIVO* METHODOLOGY

Coagulation factors and functions are similar in mice and humans, making *in vivo* models for thrombosis and bleeding a useful tool. Especially, because of the ability to create genetic manipulated mice, different *in vivo* models have been developed. *In vivo* models have provided significant insights into human hemostasis and give relevant models for evaluation of the treatment and pathophysiology of thrombosis (Emeis et al., 2007).

2.1 Arterial thrombosis model (paper II)

Arterial thrombus formation models, e.g. generated by mechanical disruption, laser-induced, photochemical-induced or ferric chloride ($FeCl_3$)-induced injury to the vessel wall are not physiologic, however have provided insights in thrombotic processes and are relevant tools for the validation of new thrombolytic, anticoagulant and anti-platelet drugs (Bonnard & Hagemeyer, 2015). In paper II we measured the blood flow in the carotid artery of mice, which were injected intravenously with PPX_Δ12 or PPX, after $FeCl_3$ challenge.

The $FeCl_3$-induced model is a commonly used thrombosis model in mice because this model is relative simple and effective. The mechanisms involved in $FeCl_3$-induced thrombosis are

not fully understood, though it seems that $FeCl_3$ generates free radicals that cause injury to the vessel wall, which induces platelet adhesion and activation. The model is dependent on the interaction of platelets, thrombin, and blood flow (Lockyer & Kambayashi, 1999). One limitation is that this model is induced in healthy vessels while thrombosis is mostly occurring in diseased/atherosclerotic vessels. Furthermore, this model is not suitable for studying long-term development of the disease. Besides these limitations the $FeCl_3$ model is shown to be relevant to the study of human thrombosis. The composition of the thrombi are similar and since arterial thrombosis is assumed to be started by the oxidation of lipoproteins the $FeCl_3$ model mimics the pathophysiology of human thrombosis more than a mechanical, laser or photochemical induced injury (Bonnard & Hagemeyer, 2015).

2.2 Pulmonary embolism model (paper II and IV)

Murine venous thrombosis models have been useful in explaining the molecular and cellular determinants of venous thrombosis. To test the effect of antithrombotic agents models inducing pulmonary embolism, a life-threatening complication of venous thrombosis, are often used. A pulmonary embolism can be provoked by injection of exogenous clots, by photochemical/$FeCl_3$ -induced injury or by activation of platelets/coagulation. The advantage of platelet/coagulation induced pulmonary embolism is that it is simple and inexpensive. However in this setting pulmonary embolism results in lethal thromboembolism and is not directly related to venous thrombosis, and therefore does not exactly imitate venous thrombosis in humans (Miao et al., 2010). Because we wanted to analyze if PPX_Δ12 or PPX interfered with platelet-induced thrombosis, we used a platelet activator to challenge the mice. Thrombosis was induced by intravenous injection of collagen and epinephrine into the vena cava, which induces widespread platelet activation followed by lethal pulmonary thromboembolism.

2.3 Tail-bleeding assay (paper II)

Tail-bleeding assays are used as an *in vivo* assessment of hemostatic action of platelets in mice. However, the used strain, sex and age of the mice, tail injury method and anesthetics impact the bleeding time resulting in large variability in data. Since the tail bleeding time assay does not require sophisticated equipment it is one of the common used models (Greene et al., 2010). Taken this in consideration we used a standardized method to cut the tail, age/sex matched mice for the control and treated group and two methods to measure the bleeding time. Mice with similar bleeding times do not always have similar amounts of blood loss (Liu et al., 2012), therefore, we also included a measurement of blood loss.

RESULTS AND DISCUSSION

1. INVESTIGATING LOCALIZATION OF POLYPHOSPHATE ON PROCOAGULANT PLATELETS (PAPER I AND II)

The role of polyP in coagulation has been studied at various points in the extrinsic, intrinsic, and common pathway. Furthermore, polyP exerts different effects on blood clotting, depending on polymer size (Smith et al., 2010). In mammalians, platelets appear to be the main source of procoagulant polyP and they release soluble short-chain polyP of 70-75 phosphate units (Ruiz et al., 2004). This soluble short-chain polyP has a limited capacity in activating FXII whereas long-chain polyP is a very potent FXII activator (Smith et al., 2010). However over the past decades, several studies have reported that activated platelets promote coagulation in a FXII-dependent manner (Back et al., 2010, Johne et al., 2006, Muller et al., 2009, Nielsen et al., 2005, Walsh, 1972, Walsh & Griffin, 1981), suggesting that FXII activation by platelet polyP mediates thrombosis *in vivo* in a yet undefined mechanism.

1.1 Only a small portion of polyP is released after platelet activation

PolyP is found in the dense granules of platelets and previous studies working with platelet polyP (Muller et al., 2009, Smith et al., 2010) suggest that these soluble polyP polymers are released. We analyzed the amount of polyP released into the supernatant by activated platelets. We activated platelets with collagen or buffer, and incubated the supernatant with *E. coli* exopolyphosphatase (PPX). The difference in monophosphate in platelet supernatants between non-activated and activated platelets provides a measure for released soluble polyP (**Figure 9**).

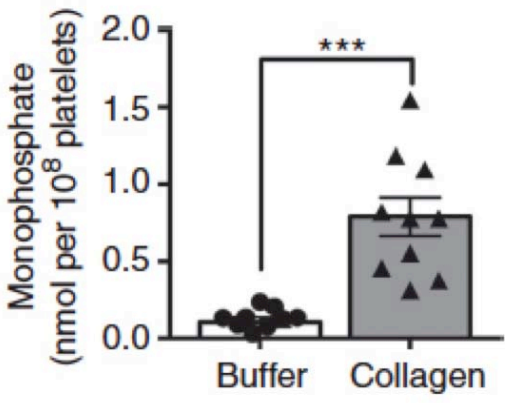

FIGURE 9. Released polyP in activated platelet supernatant.
Human platelets were incubated with collagen or buffer spiked with prostaglandin E1. Platelets were pelleted and the supernatant was digested with PPX. Monophosphate was determined by malachite green-based phosphate assay kit. Mean ± SEM, n=10, ***p<0.001 by student's *t-test*. Modified with permission (Labberton et al., 2016).

We found that collagen-activated platelets released 0.79 ± 0.12 nmol polyP/10^8 platelets. Our findings are consistent with previous studies that found 0.74 ± 0.08 nmol secreted polyP/10^8 activated platelets (Ruiz, Lea et al., 2004). Murine platelets contain about ~30 nmol total polyP/10^8 platelets (Ghosh, Shukla et al., 2013, all data are given in monophosphates). Assuming that mouse platelets are similar to human platelets, only a minor portion of platelet polyP (<5%) is released into the supernatant upon activation. Therefore, we decided to investigate the localization of the remaining polyP in platelets.

1.2 PolyP in platelets exists in two different pools

Platelet polyP can be isolated using the phenol-chloroform extraction method (Kumble & Kornberg, 1995). This method selects for water-soluble polyP while water-insoluble, e.g., long-chain, aggregated or cell-bound polyP are discharged during the isolation procedure. Using the phenol-chloroform extraction method, previous studies (Muller et al., 2009, Smith et al., 2010) purified small quantities of short-chain (60-100 monomers) soluble polyP from the supernatant of a large amount of activated platelets (Muller et al., 2009, Smith et al., 2010). These earlier data are consistent with our current findings that only a small portion of platelet-derived polyP is released into the supernatant.

However when we purified polyP from whole platelet lysates using an anion exchange method (Werner, Amrhein et al., 2007), we unexpectedly found that platelets also contain a significant amount of long-chain polyP (chain length >250, **Figure 10a**). To confirm that the isolated material was polyP, it was incubated with alkaline phosphatase, an enzyme that cleaves polyP.

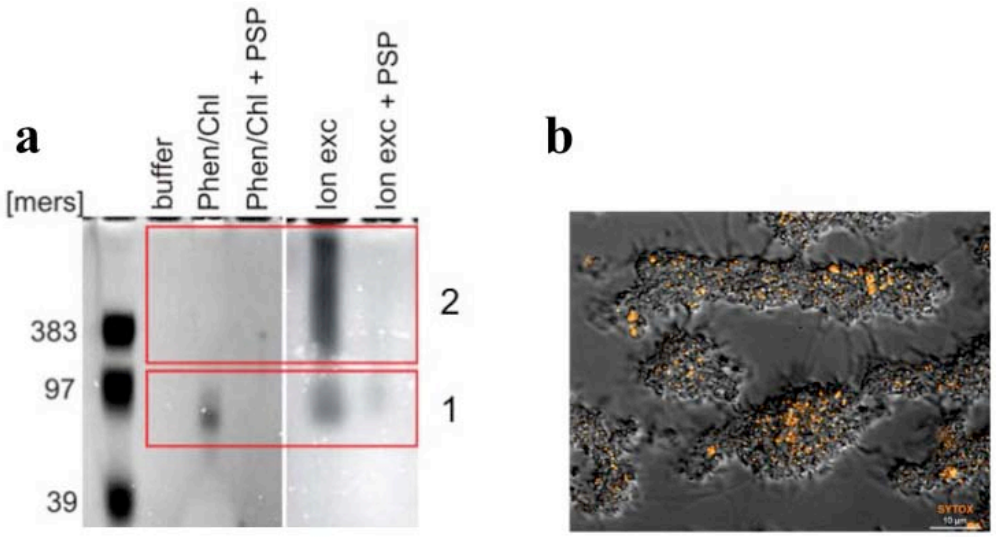

FIGURE 10. Platelet polyP exists in two pools and retains on platelet surfaces
(**a**) Gel electrophoresis of platelet polyP isolated from platelet lysates using the phenol-chloroform extraction (Phen/Chl) method or by an anion-exchange method (Ion exc). PolyP was visualized by negative DAPI-staining. PSP indicates phosphatase treatment prior to separation. Synthetic polyP with mean chain lengths of 39, 97 and 383 serves as molecular size standard. Number 1 denotes short-chain molecules (60-100 chain length) and 2 shows long-chain polyP (chain length >250). (**b**) Cross section images of platelet aggregates, formed by perfusing citrated whole blood over immobilized collagen. PolyP is stained with SYTOX orange.

The purification data suggest that short-chain, soluble polyP is released from procoagulant platelets into the supernatant and that the majority of platelet polyP is long-chain and insoluble. Solubility of polyP depends on the chain length of the polymer and its counter anion. PolyP precipitates in the presence of divalent metal cations forming polyP nanoparticles (Donovan et al., 2014). Platelet dense granules contain, besides polyP, large amounts of calcium (Holmsen & Weiss, 1979) and polyP thus may precipitate on the platelet surface into nanoparticles upon secretion. To analyze if polyP retains on the surface of platelets, platelet aggregate formation in whole blood was performed in the presence of

SYTOX orange to stain for polyP. SYTOX positive structures started to form during degranulation of the platelets, and remained present on the platelet surface, suggesting that polyP is exposed and retained on the cell surface (**Figure 10b**).

1.3 Platelet polyP nanoparticles strongly activate FXII

PolyP nanoparticles exhibit different procoagulant properties than the molecularly dissolved polymer (Donovan et al., 2014). For instance, short-chain polyP conjugated to colloidal gold nanoparticles activates FXII, with a potency equivalent to that of long-chain polyP (Szymusiak et al., 2016). PolyP nanoparticles formed in platelets can be the natural anionic contact surface needed for activation of FXII.

We compared the capacity of polyP, extracted by the two methods, to activate FXII. Platelet polyP isolated by anion exchange, strongly activated FXII, while soluble short-chain polymers (isolated by phenol-chloroform) gave a minor activation (**Figure 11a**). We subsequently investigated whether platelet polyP nanoparticle formation with Ca^{2+} happens and if this influences the capacity to activate FXII. Surprisingly, the FXII-activating potential of platelet polyP (isolated by anion exchange) was fully abrogated in the presence of EDTA (a chelator of divalent metal ions, **Figure 11b**). These experiments cumulatively suggest that in contrast to short soluble polyP, platelets contain another pool of membrane-associated polyP nanoparticles that can potently activate FXII.

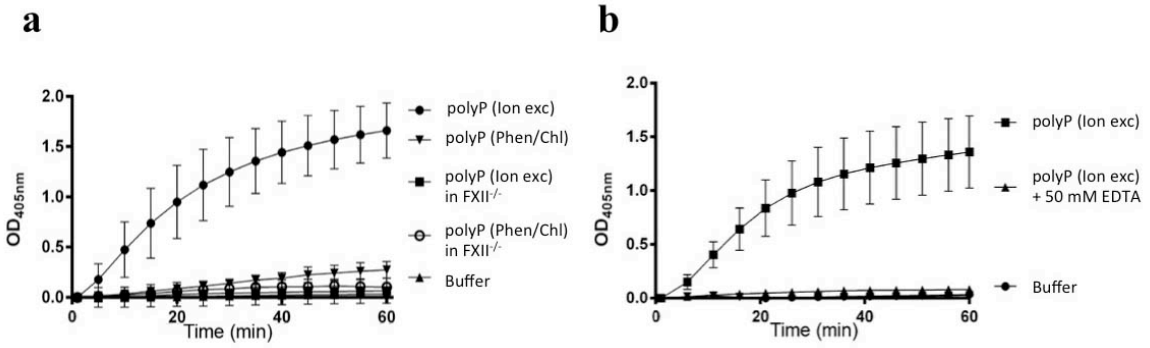

FIGURE 11. Platelet polyP nanoparticles are a potent FXII activator
(**a**) FXII activation, triggered by platelet polyP isolated by the two different methods in normal and FXII-deficient plasma. (**b**) Platelet polyP-triggered FXII activation in the absence or presence of EDTA.

2. TARGETING PROCOAGULANT POLYPHOSPHATE *IN VIVO* (PAPER II AND IV)

The role of platelet polyP in hemostasis and thrombosis makes targeting polyP an attractive and novel strategy to interfere with thrombosis. We were interested in a selective non-toxic polyP targeting strategy, which does not interfere with bleeding, as a proof-of-concept approach for preventing thrombosis. We based the strategy on the exopolyphosphatase of *E.coli* (PPX), a highly processive polyP-degrading enzyme (Akiyama et al., 1993).

2.1 PPX specifically degrades polyP

Several characteristics of PPX distinguish it from the alkaline phosphatases. Most importantly, PPX has a strong preference for long-chain polyP and fails to act on smaller sizes (shorter than 15 phosphate units) of polyP (Akiyama et al., 1993). To analyze if PPX is specific for polyP and does not cleave the phosphoanhydrid bonds of ATP and ADP, or the phosphodiester bonds of the nucleic acids DNA and RNA, we compared the phosphatase activity of PPX towards polyP, ATP, ADP, DNA and RNA.

A time dependent degradation of synthetic polyP showed that 10 µg ml^{-1} PPX degraded polyP (**Figure 12a and b**), where 8 minutes incubation was sufficient for a significant reduction of long-chain polyP (32 ± 9% reduction). Inhibition of the PPX by heparin was seen as a ≥ 80% increase in remaining polyP after 128 minutes of incubation.

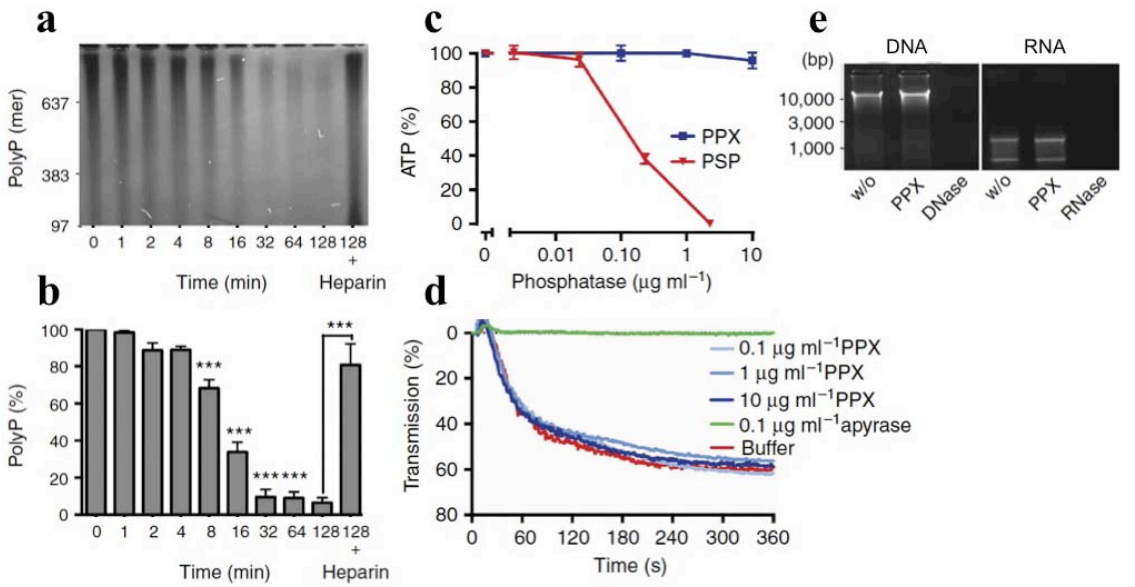

FIGURE 12: PPX specifically degrades polyP.

(**a, b**) Time-dependent hydrolysis of polyP by PPX. Long-chain (LC) polyP was incubated with 10 µg ml^{-1} PPX. Aliquots were taken at indicated time-points, resolved on 10% urea-polyacrylamide gels and visualized by negative DAPI staining. Synthetic polyP with mean chain length of 39, 97, 383 and 637 phosphates were loaded as molecular size standard. PolyP incubated with PPX in the presence of the inhibitor heparin is shown in the last lane. Bars (**b**) are mean ± SEM, from four independent experiments, ***p<0.001 *vs.* 0 min by one-way analysis of variance (ANOVA) and by Student's *t* test *vs.* heparin addition. (**c**) ATP was incubated for 30 min with increasing concentrations of PPX or shrimp alkaline phosphatase (PSP) and quantified using a luciferase-based bioluminescence assay. Data are mean ± SEM, from three independent experiments. (**d**) ADP was treated for 30 min with buffer, apyrase or increasing concentrations of PPX. Platelet aggregation in human platelet-rich plasma stimulated by the reaction mixtures. Representative curve of n=4. (**e**) DNA and RNA were treated with buffer (w/o), PPX, DNase or RNase respectively and resolved on agarose gels. Modified with permission (Labberton et al., 2016).

In contrast to polyP, PPX does not degrade the phosphoanhydrid bonds of ATP (< 7% decrease), while alkaline phosphatase dose-dependently hydrolyses ATP (**Figure 12c**). To analyze the phosphatase activity on ADP, the effect of preincubation of ADP with PPX on ADP-induced platelet aggregation was analyzed. PPX did not interfere with ADP induced platelet aggregation in platelet rich plasma, while the ADP hydrolyzing enzyme apyrase completely abolished ADP induced platelet aggregation (**Figure 12d**). In addition to ATP and ADP, DNA or RNA incubation with PPX did not break down their sugar-phosphate backbone, while the nucleic acid degrading enzymes DNase and RNase led to complete DNA and RNA degradation, respectively (**Figure 12e**). Since PPX specifically degrades polyP and does not interfere with other natural phosphate containing compounds it is a perfect enzyme to specifically target polyP.

Two independent groups solved the molecular structure of PPX and different hypotheses on how polyP interacts with PPX are proposed (Alvarado et al., 2006, Rangarajan et al., 2006). PPX is composed of four distinct domains and multiple polyP binding sites are identified responsible for the recognition of polyP. The structure of the polyP-PPX complex is not known and we were interested in determining the part of PPX that specifically interacts with polyP.

2.2 PPX deletion mutants

To examine the contribution of single domains of PPX to polyP binding, we created fourteen deletion mutants lacking different domains of PPX. These mutants, linked to an N-terminal 6xHis tag were created by using site directed mutagenesis. A scheme of the PPX and the created mutants is shown in **Figure 13**. The PPX and all fourteen mutants were expressible in *E.coli* and resulted in soluble products.

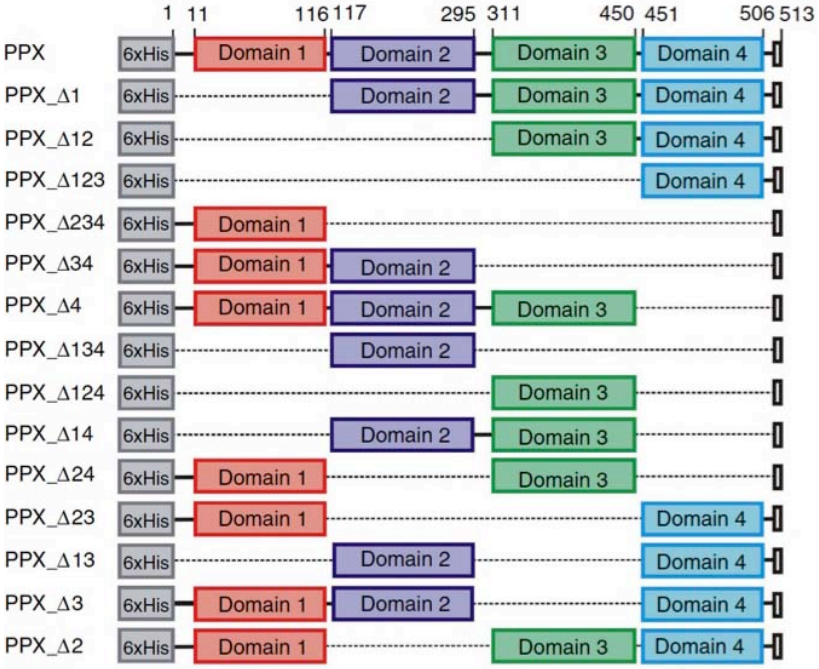

FIGURE 13: PPX deletion mutants.

Scheme of full-length PPX and PPX deletion mutants lacking various domains. Dark C-terminal squares represent the stop codons, and numbers on top indicate residues. All constructs were fused to an N-terminal 6xHis-tag. Modified with permission (Labberton et al., 2016).

2.3 PPX_Δ12 selectively binds to polyP

A binding assay was established to study the binding capacity of the generated mutants to polyP. PolyP, covalently coupled to the amine-derivated polystyrene surface of a 96 well plate by using the crosslinking agent EDAC (Choi et al., 2010), was used as the capture antigen and bound mutant was detected by a monoclonal anti 6xHis-tag antibody. PPX (set as 1, **Figure 14a**) bound with the highest affinity to polyP. The relative binding capacity of the other created mutants was reduced for mutant PPX_Δ12, mutant PPX_Δ1 and mutant PPX_Δ2, whereas binding of the other mutants tot polyP was essentially identical to background. These findings are consistent with the notion that domain 3 and 4 play a pivotal role in the binding of polyP to PPX (Bolesch & Keasling, 2000). We also pre-incubated PPX_Δ12 with polyP as a control, and under these conditions the binding of PPX_Δ12 to the immobilized polyP was completely inhibited, confirming the specificity of mutant PPX_Δ12 to polyP.

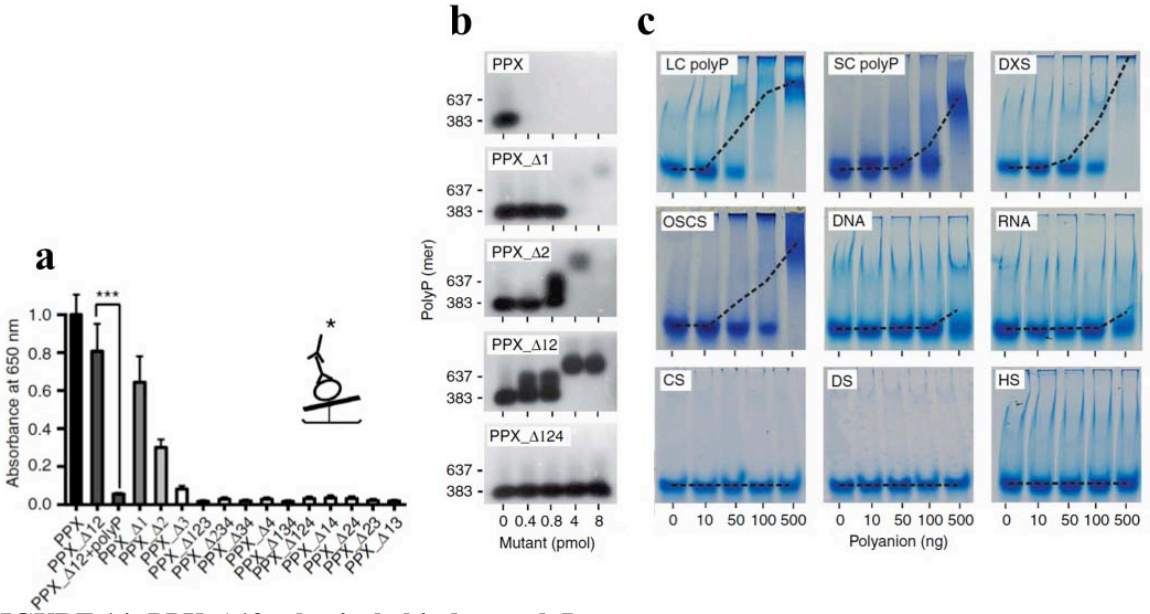

FIGURE 14: PPX_Δ12 selectively binds to polyP.

(**a**) Binding of PPX and the deletion mutants to polyP. Immobilized polyP was incubated with purified full-size PPX, PPX deletion mutants or PPX_Δ12 pre-incubated with polyP. Bound PPX variants were detected using an antibody against the 6xHis-tag, an HRP-coupled secondary antibody and substrate reaction. The cartoon shows the ELISA setup with ▬ = polyP, ⬭ = PPX variant, 人 = 6xHis-tag antibody and ⊀ = HRP-coupled detection antibody. Shown are relative amounts of PPX variants binding to polyP. Data blotted relative to full-size PPX, set to 1.0. Mean ± SEM, n=4, ***p<0.001 by Student's t test. (**b**) Gel-mobility shift assay of full-size PPX, PPX_Δ1, PPX_Δ2, PPX_Δ12 and PPX_Δ124 binding to polyP. Increasing concentrations of PPX mutants were incubated with polyP and reaction mixtures were resolved on agarose gels. PolyP was visualized with DAPI-negative staining. (**c**) PPX_Δ12 was incubated with increasing concentrations of various polyanions including dextran sulfate (DXS), over sulfated chondroitin sulfate (OSCS), chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS). PPX_Δ12/polyanion complexes were dissolved on urea-polyacrylamide gels and PPX_Δ12 protein was stained with Coomassie brilliant blue. The dashed line gives PPX_Δ12/polyanion complex formation assessed by densitometric scans. Modified with permission (Labberton et al., 2016).

In the above-mentioned method established for polyP binding, polyP is protected from PPX degradation because of phosphoramidate linkage by using EDAC (Choi et al., 2010). Therefore a gel shift assay was used to define mutant binding to polyP, where the phosphatase activity of the different mutants could be assessed simultaneously. Increasing concentrations of the mutants were incubated with a constant concentration of polyP. Gels

shift assays (**Figure 14b**) show that PPX, PPX_Δ1 and PPX_Δ2 bind to and hydrolyze polyP. In contrast mutant PPX_Δ12 did not hydrolyze polyP, yet bound to polyP as seen by the shift. Further deletions of domain 3 or 4 resulted in loss of binding to polyP as seen in mutant PPX_Δ124. Since PPX_ Δ12 is the mutant that bound to polyP but did not hydrolyze the polymer we chose it for further use.

The interaction between PPX and polyP is mostly driven by charge (Rangarajan et al., 2006). We were therefore interested in the binding of PPX_Δ12 to other negatively charged molecules. Electrophoretic mobility shifts confirmed a strong interaction of PPX_Δ12 with long-chain and short-chain polyP (**Figure 10c**). Additionally PPX_Δ12 bound to the non-physiological substrates dextran sulfate and over sulfated chondroitin sulfate while PPX_Δ12 failed to interact with other natural occurring polyanions such as chondroitin sulfate, DNA, RNA and heparan sulfate.

2.4 PPX and PPX_Δ12 specifically interfere with polyP-induced coagulation

A dual strategy of polyP degradation by the specific polyP-degrading enzyme PPX and inhibiting polyP by binding with the high affinity mutant PPX_Δ12 was chosen to interfere with polyP-induced coagulation. We assessed the ability of PPX and PPX_Δ12 to inhibit the procoagulant activity of different physiological and non-physiological contact activators. Real time thrombin formation assays showed that both PPX and PPX_Δ12 dose-dependently reduced total and maximum thrombin formation and prolonged the lag time in plasma stimulated with the physiological contact activators polyP (**Figure 15a and b**). Consistently, PPX_Δ12 blocked the procoagulant activity conferred by polyP isolated from a prostate cancer cell line (**Figure 15c**). In contrast, PPX and PPX_Δ12 had no measurable effect on thrombin formation by the non-physiological FXII activator silica (**Figure 15d**), the physiological activator DNA (**Figure 15e**) or the physiological activator of the extrinsic pathway, tissue factor (**Figure 15f**). An advantage compared to the polyP binding PdsP15 (Alvarenga et al, 2013) and PAMAM (Jain et al., 2012), which also bound to other polyanions and interfered with silica- and nucleic acid-driven plasma clotting.

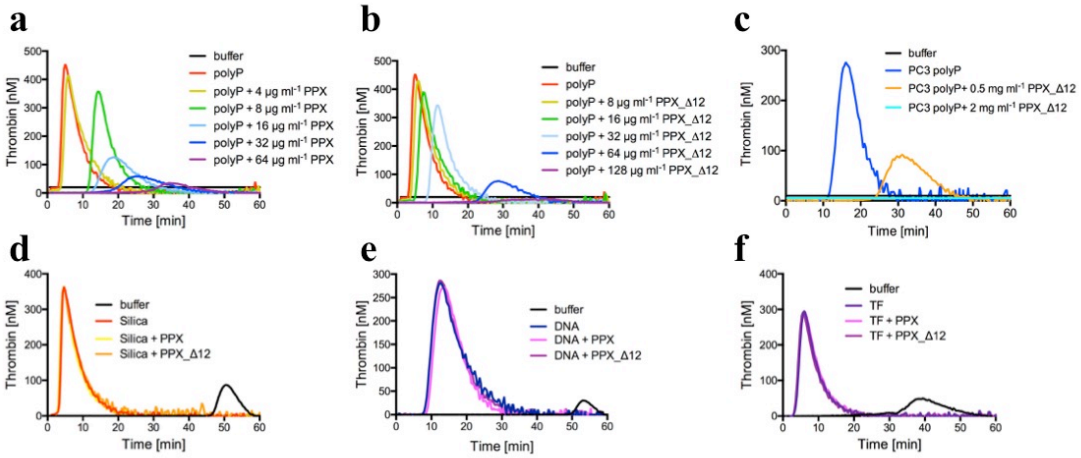

FIGURE 15: PPX and PPX_Δ12 specifically interfere with polyP-induced coagulation.
Real-time thrombin generation in the absence or presence of PPX or PPX_Δ12 in PPP stimulated with polyP (**a** and **b**), PC3 cell polyP (**c**), silica (**d**), DNA (**e**) or tissue factor (TF; **f**). Representative thrombin generation curve of *n*=6 is shown. Modified with permission (Labberton et al., 2016).

2.5 PPX and PPX_Δ12 specifically interfere with thrombus formation under flow

Collagen is exposed at sites of vascular injury and plays a main role in platelet induced coagulation. We therefore studied interference of PPX and PPX_Δ12 with collagen-dependent thrombus formation under flow. Citrate-anticoagulate blood from humans was recalcified and perfused at an arterial (**Figure 16a**) or venous (**Figure 16b**) shear rate over immobilized collagen fibers. In buffer-treated samples, platelets adhere to collagen fibers and fibrin was formed resulting in 34 ± 2% and 39 ± 3% surface covered area, under arterial and venous shear rates respectively. PPX and PPX_Δ12 dose-dependently reduced thrombus formation in both arterial and venous flow significantly and almost abolished thrombus formation (<10% surface covered area) at a concentration of 2 mg ml^{-1}.

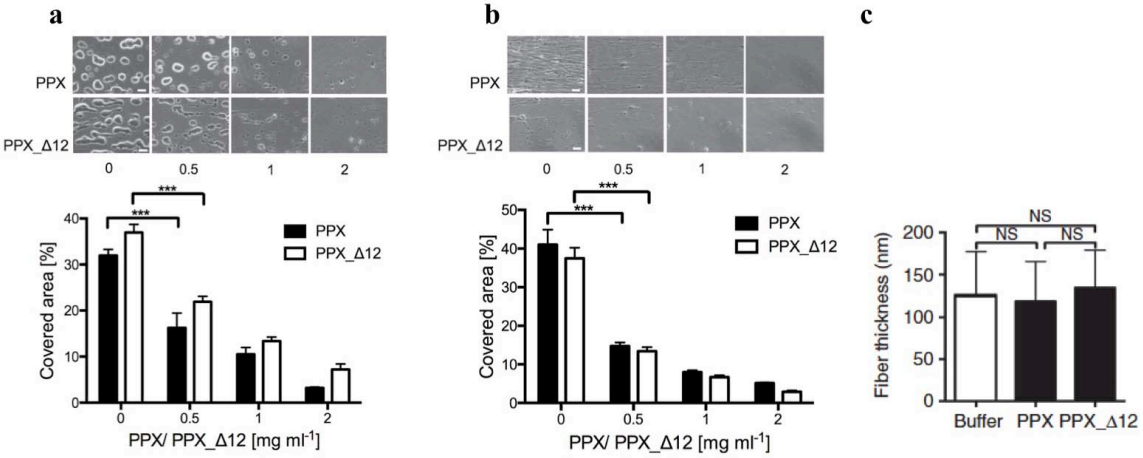

FIGURE 16: PPX and PPX_Δ12 specifically interfere with thrombus formation under flow.
Human citrated whole blood, readjusted to physiologic Ca^{2+} and Mg^{2+} concentrations, was perfused over a collagen-coated surface at an arterial (**a**) or venous (**b**) shear rate. Representative phase-contrast images of thrombi formed during perfusion in the absence or presence of indicated PPX or PPX_Δ12 concentrations. Scale bars are 20 µm. Columns give the percentage of surface area covered by thrombi. Mean ± SEM, from four independent experiments, ***p<0.001 by one-way ANOVA. (**c**) Fiber thickness measured of thrombi formed in collagen-dependent coagulation under flow from scanning electron micrographs of 25 fibers in 3 representative areas. Mean ± SEM, NS = non-significant by one-way ANOVA. Modified with permission (Labberton et al., 2016).

In a purified system polyP affects clot structure by enhancing fibrin polymerization and attenuating binding of fibrinolytic protein to fibrin (Mutch et al., 2010, Smith et al., 2006). Synthetic polyP increases final clot turbidity and fibrin fiber thickness in a Ca^{2+} dependent way (Smith & Morrissey, 2008b). However, other studies using polyP do not show an effect on the average fiber diameter (Mutch et al., 2010) or on modulation of fibrin clot structure (Smith et al., 2010). Our data on fibrin fiber thickness of thrombi formed in collagen-dependent coagulation under flow, showed no significant change in the diameter of fibrin fibers in the presence of PPX and PPX_Δ12 (**Figure 16c**). The contradictory results could be explained by differences in polyP concentrations. We used a whole blood-flow system with physiological polyP concentrations, which are in the submicromolar range (Lorenz et al., 1997), and lower than used by Smith *et al.* (Smith & Morrissey, 2008b). Furthermore the preincubation step of fibrinogen, Ca^{2+}, and polyP, which was necessary for the increase in fiber thickness to be observed, (Smith & Morrissey, 2008b) is missing. Finally, pyrophosphate and to a lesser extent monophosphate and triphosphate abrogate the ability of

polyP to enhance fibrin clot structure (Smith et al., 2010). Plasma and platelet dense granules contain substantial quantities of pyrophosphate (Holmsen & Weiss, 1979), which is released after platelet activation (Fukami et al., 1980). Indicating that specific targeting of polyP in a physiological setting has no influence on fibrin structure.

Fibrin clots generated from mice with significantly reduced platelet polyP levels (*IP6K1*$^{-/-}$ mice) have homogenous web-like clot architecture, whereas thicker fibrin fibrils and tight fibrin aggregates were observed in wild type (WT) clots (Ghosh et al., 2013). This seems to indicate that the presence of polyP within a clot alters its structure. However *IP6K1*$^{-/-}$ mice have multiple other defects e.g. lower body weight, reduced insulin levels, and defective spermatogenesis. This indicates important roles for *IP6K1* and inositol pyrophosphates in several other physiological functions (Bhandari et al., 2008) and not only a reduction in platelet polyP, while our polyP targeting strategies specifically target polyP.

2.6 Targeting polyP interferes with thrombosis but spares hemostasis

We next examined the *in vivo* activity of PPX and PPX_Δ12. We challenged mice in a model of lethal pulmonary thromboembolism by infusing collagen-epinephrine into the inferior vena cava. Seven out of eight buffer treated WT mice died within 5 minutes of collagen application. In contrast, animals treated with PPX or PPX_Δ12 were significantly protected from platelet-induced pulmonary embolism, with respectively six or four out of eight surviving the challenge (**Figure 17a**).

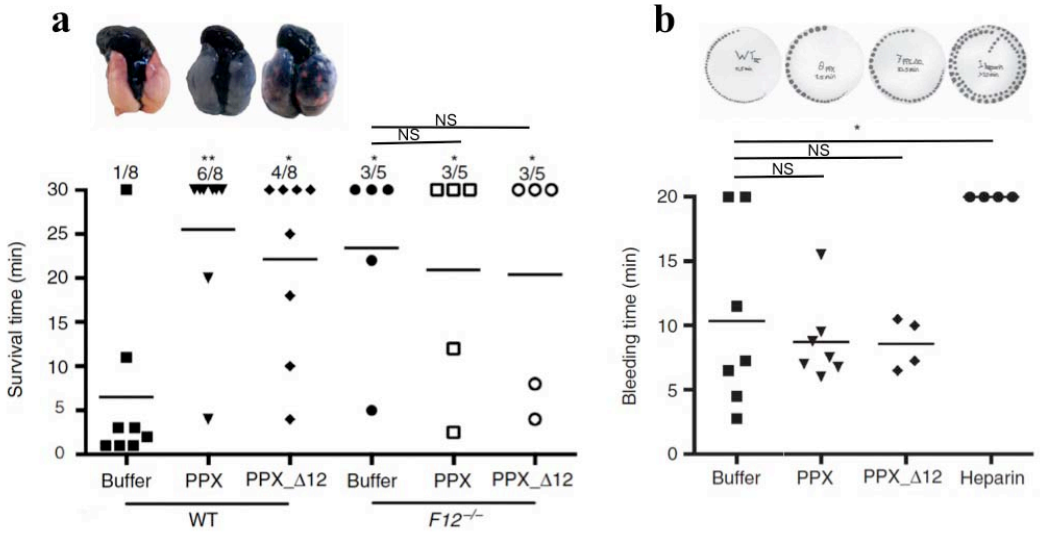

FIGURE 17: Targeting polyP interferes with thrombosis but spares hemostasis.
(**a**) Pulmonary embolism induced by intravenous infusion of collagen-epinephrine. The survival time of WT or *F12*$^{-/-}$ mice pre-treated with buffer, PPX or PPX_Δ12 was monitored. Mortality was assessed in each group of mice, and animals alive 30 min after challenge were considered survivors. Collagen-epinephrine-challenged mice were intravenously infused with Evans blue shortly after the onset of respiratory arrest while the heart was still beating or after 30 min for those animals that survived. Lungs were excised and perfusion defects were analyzed. Occluded parts of the lungs remain their natural pinkish color. (**b**) Wild-type mice were pre-treated with buffer, PPX, PPX_Δ12 or heparin and tail-bleeding times were analyzed by gently absorbing blood with a filter paper. Each symbol represents one animal; bars within each column indicate the mean. **p<0.01, *p<0.05 *vs.* buffer-treated WT by one-way ANOVA, NS = non-significant. Modified with permission (Labberton et al., 2016).

Consistent with earlier data (Renne et al., 2005) *F12*$^{-/-}$ mice were also protected in this model, and surprisingly infusions of PPX and PPX_Δ12 did not give an additional survival advantage in *F12*$^{-/-}$ mice, showing that specific targeting of polyP protects from platelet-induced pulmonary embolism in a FXII dependent manner. To confirm that death from collagen challenge was due to pulmonary embolism, we analyzed the degree of vascular occlusion in the lungs by intravenous perfusion with Evans blue dye (Hamilton et al, 2004). Collagen challenge in buffer treated WT mice initiated lung vessel occlusion visualized by disturbed perfusion of Evans blue. In contrast, lungs of mice treated with PPX and PPX_Δ12 were less affected by embolic events and lungs were mostly blue.

Because bleeding is a major problem of currently used anticoagulants we were interested in the bleeding times of PPX- or PPX_Δ12-treated mice. We determined bleeding time by tail clipping. The time to stop bleeding of PPX- or PPX_Δ12-treated mice did not significantly differ from buffer-treated mice (**figure 17b**). In contrast, heparin-treated mice exhibited significantly prolonged bleeding time, showing that selective targeting of polyP blocks thrombosis without affecting hemostasis.

Mechanistically, we show here that thromboprotection conferred by targeting of polyP is mediated by interference with FXII activation *in vivo*. It is shown *in vitro* that polyP contributes to other procoagulant reactions involving FV, TFPI and fibrin. However, in contrast to FXII deficiency, low FV (Ang et al, 2009), elevated TFPI (Maroney & Mast, 2015) or defective fibrin structure (Casini et al, 2015) are associated with increased bleeding. Targeting polyP with PPX or PPX_Δ12 does not alter hemostasis arguing against a significant role of polyP driven FXII-independent mechanisms for *in vivo* coagulation.

Opposed to previously used polyP inhibitors, our strategies have a high specificity for polyP. Earlier studies show that inhibitors of polyP, including polycationic compounds, salivary sand fly proteins and nuclear-acid based polymers, can block the procoagulant effects of polyP, both *in vitro* and *in vivo* (Alvarenga et al., 2013, Jain et al., 2012, Smith et al., 2012, Travers et al., 2014). However none of the existing polyP inhibitors were specific since other *in vivo* activities were influenced as well. One example is the cationic PAMAMs (Jain et al., 2012, Smith et al., 2012), which are shown to be hemolytic (Malik et al., 2000) and cytotoxic (Roberts et al., 1996). Furthermore these dendrimers induce fibrinogen aggregation (Travers et al., 2014).

The recent developed universal heparin reversal agents (UHRAs) show increases in TF-initiated plasma clotting. Moreover the concentration needed to inhibit arterial thrombus formation increased the bleeding time aproximately three times (Travers et al., 2014). The mechanism of UHRAs interference with hemostatic mechanisms is not precisely known. This increase in bleeding time could be a result of unspecific binding of the UHRAs, comparable to the positively charged polypeptide, protamine. Protamine administration is associated with increased bleeding, since protamine inhibits FV activation by charge-mediated interactions (Ni Ainle et al., 2009). Besides inhibition of polyP with an anionic polymer-binding mechanism we provide a specific enzymatic approach to inhibit procoagulant polyP

activities. All the previously used polyP inhibitors act by stoichiometrically binding where the amount of binding sites is the limitation. An enzyme can continue its action after hydrolyzing its substrate and is therefore not limited. This can be an advantage especially in platelet rich thrombi were presumably, local polyP concentrations are high (Morrissey, 2012).

3. ANALYSIS OF POLYPHOSPHATE ON CELLS AND IN HUMAN SAMPLES (PAPER III, IV)

Due to the recent recognition of the inorganic polymer polyP as a procoagulant platelet-derived mediator, we examined the use of our specific polyP-binding probe, PPX_Δ12, to analyze polyP on cells and in human samples

3.1 PPX_Δ12 probes polyP on prostasomes and prostate cancer cells

Thrombosis is a common complication of malignancies in patients with cancer. The mechanism underlying cancer-associated thrombosis is considered to be increased TF expression on cancer cells and cancer cell-derived membrane vesicles (Dicke et al., 2015). However, Nickel *et al.* found that prostasomes from prostate cancer (PC) patients initiate lethal pulmonary embolism via the intrinsic pathway of coagulation. Consistently FXIIa inhibition provided safe protection (Nickel et al., 2015). Therefore we wondered if polyP/FXII-triggered coagulation plays an important role in PC-associated thrombosis and if prostasomes from prostate cancer patients contain polyP on their surface.

We conjugated amine-reactive Alexa594 to PPX_Δ12 to generate fluorescently labeled PPX_Δ12 and used this fluorescent probe to detect polyP. PolyP localized to the surface of prostasomes and co-localized with the prostasome cell surface marker CD63 (**Figure 18a**, upper panel). Furthermore, incubation of prostasomes with human plasma led to FXIIa formation and polyP co-localized with FXIIa (**Figure 18a**, lower panel). To quantify polyP expression on the surface of various types of prostasomes an ELISA using PPX_Δ12 was developed. Prostasomes were immobilized to a 96-well plate and polyP on their surface was detected by using PPX_Δ12, 6xHis-tag antibody, HRP-coupled antibody and TMB substrate reaction (**Figure 18b**). PolyP content on the different types of prostasomes correlated with the procoagulant activity of the respective prostasomes (Nickel et al., 2015).

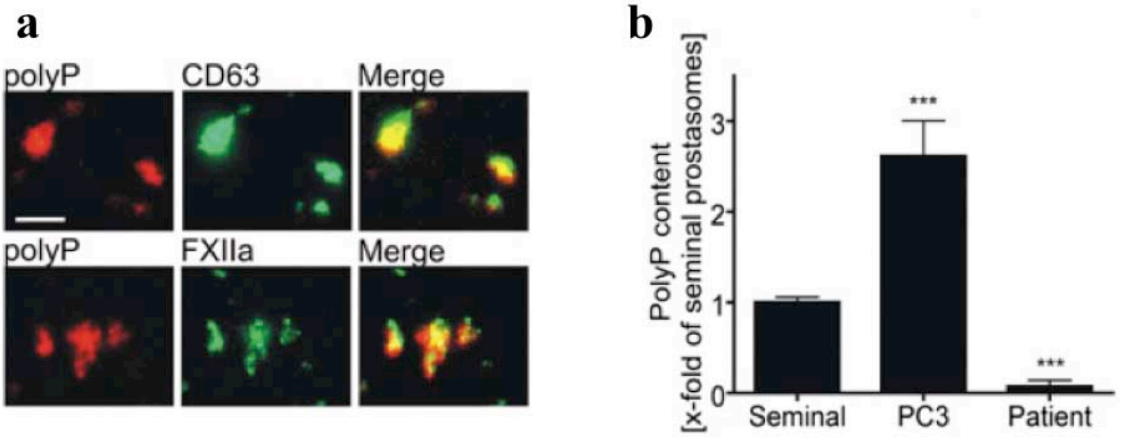

FIGURE 18. Prostasomes and PC cells expose procoagulant polyP.
(a) Fluorescence images of polyP on prostasomes using Alexa 594-labeled PPX_Δ12. The polymer colocalizes with prostasome surface membrane marker CD63 and FXIIa. (**b**) PolyP detection on seminal, PC3 cell, and patient prostasomes using PPX_Δ12 binding in an ELISA. Bars represent polyP content relative to seminal prostasomes (set to 1), mean ± SEM, n=3. *** $p<0.001$ *vs.* seminal prostasomes, by one-way ANOVA. Modified with permission (Nickel et al., 2015).

3.2 PPX_Δ12 probes polyP on procoagulant platelets

Establishing polyP as a novel biomarker could offer the exciting opportunity to develop strategies for diagnostics and safe interference with both thrombotic and inflammatory diseases. To measure polyP on the surface of procoagulant human platelets we used PPX_Δ12 covalently coupled to amine-reactive Alexa488 to generate a directly fluorescently labeled polyP probe, PPX_Δ12-Alexa488. To assess whether PPX_Δ12 had the capacity to probe for polyP on platelets we used flow cytometry, which is the standard method to analyze platelet surface markers such as the platelet integrin $\alpha_{2b}\beta_3$ and the glycoprotein complex Ibα/β-IX-V (van Velzen et al., 2012).

To establish the method we incubated resting platelets with increasing concentrations of synthetic short-chain polyP (SC, mean chain length of 70 units, similar to that of the soluble polymers in the supernatant of activated platelets) and long-chain polyP (LC, mean chain length 700 units). We determined differences in mean intensity of fluorescence (MFI) in the absence and presence of polyP in the Alexa488 channel of the flow cytometer. PPX_Δ12-Alexa488 binding to resting non-activated platelets before polyP incubation was low and set to 1. When platelets were incubated with LC polyP the PPX_Δ12-Alexa488-signal on platelets increased with the addition of increasing amounts of polyP (1.5 ± 0.3; 2.7 ± 0.4 and 4.7 ± 1.1-fold for 1.5, 3 and 10 mM LC polyP, respectively, **Figure 19a**).

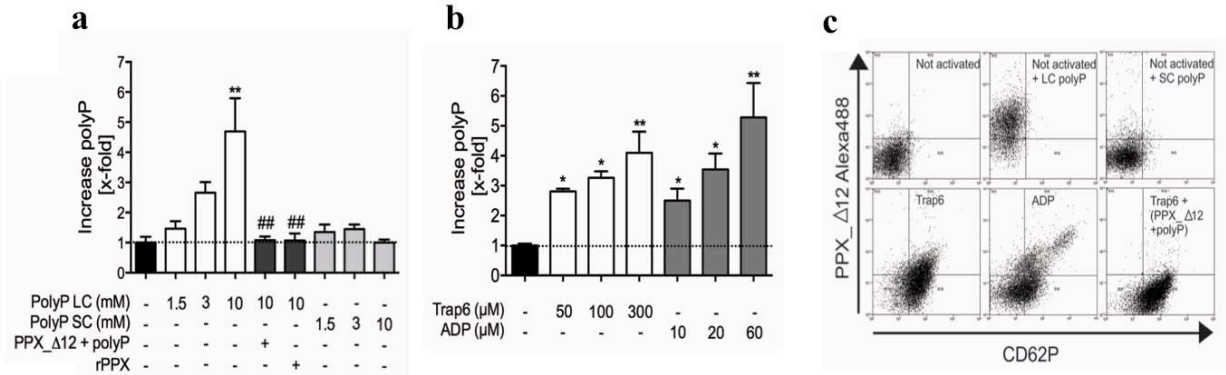

FIGURE 19. Analysis of polyP on platelet surfaces.

Non-activated human platelets were incubated with increased concentrations (1.5 - 10 mM monophosphate) of long-chain (LC) or short-chain (SC) polyP (**a**) or were stimulated with increased concentrations of trap6 or ADP (**b**). Platelets were incubated in the presence of labeled anti-CD42b (platelet marker) and anti-CD62P (P-selectin, activation marker). Alexa488-labeled PPX_Δ12 quantifies polyP. Columns give PPX_Δ12-Alexa488 signal intensity on 10 000 platelets blotted relative to buffer-incubated platelets (set to 1). For control, LC polyP-incubated platelets were treated with PPX before probing with PPX_Δ12-Alexa488. Alternatively, PPX_Δ12-Alexa488 was pre-incubated with SC polyP before probing. Mean ± SEM, n=4, * p<0.05, ** p<0.01 *vs.* buffer, ## p<0.01 *vs.* 10 mM monophosphate LC polyP only, one-way ANOVA. (**c**) Density plots from polyP on platelets incubated with long-chain (LC), short-chain (SC) polyP, trap6 (100 μM) or ADP (20 μM). Representative image of n=4 experiments is shown.

These data indicated that synthetic long-chain polyP localizes on the surface of platelets and that PPX_Δ12-Alexa488 is able to detect this interaction. In contrast, platelets incubated with increasing concentrations of SC polyP did not give an increased PPX_Δ12-Alexa488-signal. To analyze the specificity of PPX_Δ12-Alexa488 to surface bound polyP, the samples were treated with PPX to specifically degrade polyP or PPX_Δ12-Alexa488 was pre-incubated with SC polyP before incubation with the samples. The PPX_Δ12-Alexa488-signal on

platelets incubated with the highest concentration LC polyP decreased when pre-incubated PPX_Δ12-Alexa488 was used or after treatment with PPX and was close to that of buffer incubated controls.

To probe for endogenous polyP exposure on procoagulant platelets we activated platelets with trap6 or ADP. Stimulation of platelets with increasing concentrations of trap6 (50, 100 and 300 μM) was paralleled with increased PPX_Δ12-Alexa488 signal indicating polyP deposition on platelet surfaces (2.8 ± 0.1; 3.3 ± 0.2 and 4.1 ± 0.7-fold, respectively, **Figure 19b**). Similarly, ADP stimulation resulted in a dose-dependent polyP-exposure (2.5 ± 0.4; 3.5 ± 0.5 and 5.3 ± 1.1-fold for 10, 20 and 60 μM ADP, respectively). The original density plots (**Figure 19c**) show the relationship of LC- and SC polyP incubation and platelet activation (assessed by CD62P) with polyP exposure (assessed by PPX_Δ12-Alexa488).

Here, we establish a flow cytometry-based assay for polyP that monitors the polymer on platelets. The study presents the first assay to analyze polyP in human platelet-rich plasma and shows that procoagulant platelets bind polyP on their surface. Our assay offers the opportunity of analyzing a possible thrombotic biomarker in future clinical trials.

CONCLUSION AND FUTURE PERSPECTIVES

In **paper I** and **II** investigated we the nature of platelet polyP. We found that activated platelets secrete soluble short-chain polyP in solution, while they expose insoluble long-chain polyP as nanoparticles on their membranes. These platelet polyP nanoparticles have a strong capacity to activate FXII, compared to short-chain molecules, providing an explanation to how platelet-mediated FXII activation can be initiated by polyP.

The main aim of this thesis was to develop a strategy to specifically interfere with the activity of procoagulant polyP. In **paper II** and **IV** we used clotting experiments in human plasma and arterial and venous thrombosis models in mice, which revealed that both binding to and degradation of polyP provided substantial protection from thrombosis that was not associated with any increase in bleeding tendency. To our knowledge, this is the first study that shows selective inhibition of polyP and due to the specificity of the approach, our findings differ from the previously published studies. Considering the dose (300 mg kg^{-1} bw) used in the *in vivo* models, it does not directly seem appropiate to use PPX or PPX_Δ12 as a future drug in patients. However, the role of the intrinsic pathway of coagulation develops to be important in thrombosis caused by blood-contacting medical devices (Jaffer et al., 2015). Bare-metal stents and drug eluting stents stimulate adhesion and activation of platelets resulting in thrombus formation. Therefore, effective anti-platelet therapy is necessary after stent implantation (Alt et al., 2000), offering new opputunities. For future purposes, immobilizing PPX on surfaces such as stents or heart valves could be a promising strategy. As PPX is an enzyme, it will be able to degrade multiple polyP molecules, exceeding the activity of commonly used inhibitors that solely target a single protease.

In **paper III** and **IV** we demonstrate that PPX_Δ12 specifically probes polyP and that this probe can be used to quantify polyP. Furthermore, we show that long-chain polyP binds on the platelet plasma membrane and this interaction can be analyzed on human platelets in platelet-rich plasma using a flow cytometry-based assay. Analytical validation of these methods by quantifying polyP in plasma from healthy human volunteers, and in plasma of patients with thromboembolic disease can perhaps establish a novel diagnostic test to quantitate polyP in patient samples. Future investigations have to determine if polyP can be used as a novel biomarker of thrombosis and if polyP levels are relevant for thrombotic diseases. Establishing polyP as a novel biomarker offers a new opportunity to develop strategies for diagnostics and safe interference with both thrombotic and inflammatory diseases.

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